1IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of :   Axel ULLRICH
Serial No.   :   09/461,090
For   :   EGF RECEPTOR TRANSACTIVATION BY G-PROTEIN
COUPLED RECEPTORS REQUIRES METALLOPROTEINASE
CLEAVAGE OF pro HB-EGF
Filed   :   December 14, 1999
TC/A.U.   :   1634
Examiner   :   Frank Wei Min Lu
Docket No.   :   2923-347
Customer No.   :   6449
Confirmation No.   :   3321

Commissioner for Patents
P.O. Box 1450   March 14, 2008
Alexandria VA   22313-1450

APPELLANT'S APPEAL BRIEF UNDER 37 C.F.R. §41.37

Sir:

The following comprises the Patent Owner's Brief on Appeal from the Office

Action dated August 14, 2007, in which claims 40-45, 47 and 48, were finally rejected.

A Notice of Appeal was filed on November 14, 2007. This Appeal Brief is accompanied

by the required Appeal fee set forth in 37 C.F.R. § 41.20(b)(2), and is being timely filed

on March 14, 2008.

I.

REAL PARTY IN INTEREST

The owner of the above-referenced patent and the real party in interest in this

appeal is Max-Planck-Gesellschaft Zur Forderung Der Wissenschaften E.V. Munchen,

Germany.

II.

RELATED APPEALS AND INTERFERENCES

The Patent Owner is unaware of any other appeals or interferences related to the subject matter of this appeal.

III.

STATUS OF CLAIMS

The rejection of claims 40-45, 47 and 48, all of the claims under consideration in the present application, is being appealed.   Claims 44, 45 and 47 are independent with claims 40-43 and 48 depending directly from claim 47. No claims are allowed. The appealed claims are reproduced in the Appendix attached hereto.

Claims 1-39 and 46 were previously canceled.

IV.

STATUS OF AMENDMENTS

Claims 44, 45 and 47 were amended in the response to the non-final rejections, which was filed on June 4, 2007. No amendments were made in response to the final rejection mailed August 14, 2007. Therefore, it is believed that all amendments have been entered.

V.

SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention is directed to a method for modulating growth factor receptor activation by modulating G-protein mediated signal transduction (page 1, lines 7-9). Prior to the present invention, growth factor receptor transactivation was generally assumed to be exclusively mediated via intracellular signals (page 10, lines 18-19). The present inventors have found that the growth factor receptor extracellular domain has a critical function in GPCR mediated transactivation. The activation of growth-factor receptors such as epidermal growth-factor receptor (EGFR) upon GPCR stimulation, requires the receptor's extracellular domain and can be mediated via an extracellular signal pathway (page 1, lines 21-23). When a ligand activates heterotrimeric G-proteins by interaction with a GPCR, an intracellular signal results that induces the extracellular activity of a transmembrane metalloproteinase. This causes extracellular processing of a transmembrane growth factor precursor and release of the mature factor which directly or indirectly interacts with the exodomain of the EGFR leading to intracellular autophosphorylation and signal generation (page 17, lines 2-9). The inhibition of growth factor precursor processing has been found to block GPCR-induced growth factor receptor transactivation and downstream signals (page 1, lines 26-28). The present invention can be used for the treatment or prevention of diseases which are associated with pathological growth factor receptor transactivation. In particular, the present invention provides a method for preventing or treating hyperproliferative diseases such

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as tumors, thyroid hyperplasia, retinitis pigmentosa, precocious puberty, acromegaly and asthma (page 3, lines 18-29).

In the present invention, G-protein mediated extracellular signal transduction is stimulated in a cell having a growth factor receptor tyrosine kinase which is activated by said stimulation (page 13, lines 7-10). The G-protein mediated extracellular signal transduction pathway includes cleavage of a growth factor precursor (page 2, lines 24-31). The cell is then contacted with a compound which directly binds (page 3, line 15) or acts (page 3, lines 8-16) on a growth factor precursor. The growth factor receptor tyrosine kinase activation is thereby inhibited by modulating G-protein mediated signal transduction.

The present invention is also directed to a method for identifying compounds which can be used to modulate G-protein mediated signal transduction (page 6, lines 5-22) and a method for modulating growth factor receptor activation by modulating a G-protein mediated signal transduction in a cancer cell (page 16, lines 4-19).

VI.
GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The first issue on appeal is whether the invention claimed in claims 40-43, 45, 47 and 48 fails to comply with the written description requirement under 35 USC §112, first paragraph.

The second issue on appeal is whether the invention claimed in claims 44, 45

and 47 are vague and indefinite under 35 USC §112, second paragraph.

The third issue on appeal is whether the invention claimed in claims 44 and 45

can reasonably be found obvious under 35 USC §103(a) as unpatentable over Dong et

al., (Proc. Natl. Acad. Sci. USA, 96, 6235-6240, May 1999) in view of Klemke, et al.,

(The Journal of Cell Biology, 127, 859-866, 1994).

VII.
ARGUMENTS

Claims 40-43, 45, 47 and 48 comply with the written description requirement under 35 USC §112, because they recite subject matter described in the present specification.

The Examiner contends that the language "G protein mediated extracellular

signal transduction pathway which activates a growth factor receptor" is new matter.

The office action dated August 14, 2007, states that ""[A]lthough original claim 1 contains

the language "G protein mediated signal transduction" and original claim 3 contains "an

extracellular signal pathway", and page 2 lines 7-10 of the specification describes that

activation of the growth-factor receptor is mediated by its extracellular domain and via

an extracellular signal pathway, these descriptions only supports that growth-factor

receptor is mediated by its extracellular domain in G protein mediated signal

transduction"". Applicants respectfully contend that the originally filed application

supports the language "G protein mediated extracellular signal transduction pathway

which activates a growth factor receptor". Page 2, lines 5-22 clearly describe a G-

protein mediated signal transduction pathway which leads to the activation of a growth

factor receptor via its extracellular domain and an extracellular signal pathway. Original

claim 3 depends from claim 1 and recites that the activation of the growth factor receptor is mediated via an extracellular signal pathway. Original claim 1 indicates that the growth factor receptor is capable of being modulated with a modulator of G-protein mediated signal transduction.

In addition, it is shown on page 10, lines 25-32 that the GPCR ligand bound activation of the growth factor receptor does not comprise the intracellular domain. For this reason, a chimeric receptor, which contains the extracellular domain of EGFR and the intracellular domain of the PDGFR, has been activated in RAT1 cells by adding GPCR ligands. A complete PDGF receptor (extra-/intracellular domain) is in this cell type, however, it is not activated by adding GPCR ligands. Obviously, the intracellular domain of the PDGF receptor is not sufficient for the GPCR ligand bound one according to the invention. One skilled in the art would conclude that the activation has to take place extracellularly, otherwise both receptor types, i.e. chimeric and complete receptor, would be activated by the addition of GPCR ligands (see also Figure I b and I d and page 11, lines 2-16).

This correlation is confirmed in the present invention by the Examples described on page 11, lines 26-32 and page 12, lines 1-13. It is disclosed therein that the activation of a GPCR bound signal pathway in a cell induces the activation of a growth factor receptor at a second cell, which is in the immediate proximity (Figure 2a). Thus, there is an intercellular and also an extracellular signal pathway from one cell to the other. Applicants previously pointed out that the exact language used in the claims does not need to appear in the specification. MPEP §2163.02 states that the "subject matter of

the claim need not be described literally (i.e. using the same terms or *in haec verba*) in

order for the disclosure to satisfy the description requirement". MPEP §2163.07(a)

indicates that if a disclosed device inherently performs a function or has a property, the

patent application discloses that function or property even if it says nothing explicit

concerning it and the application may later be amended to recite the function without

introducing new matter. The Board of Patent Appeals and Interferences also

interpreted the written description requirement in Ex parte Holt, 19 USPQ2d 1211 (Bd

Pat App & Inter, 1991) and in Ex parte Eggleston, et al, Appeal No. 2003-2074. In Holt

the claims were directed to a component having a channel. The Examiner rejected the

claims as lacking adequate support for the channel. The Board held that the figures

illustrate a channel in accordance with the common and accepted meaning of the term.

The Board stated that "It is well established that the invention claimed need not be

described ipsis verbis in the specification in order to satisfy the disclosure requirements

of 35 U.S.C. §112". In Eggleston, the claims were directed to a method of forwarding

messages between a host system and a mobile client. The Examiner contended that

an explicit limitation in the claims was not present in the written description. The Board

stated that explicit disclosure of the claimed term is not required under 35 U.S.C. §112,

first paragraph. Applicants respectfully contend that the language "G protein mediated

extracellular signal transduction pathway which activates a growth factor receptor" is not

new matter.

The office action dated August 14, 2007 also indicates that "CRM197, a

catalytically inactive form of the diphtheria toxin, which specifically binds to proHB-EGF

and which is capable of blocking the processing of proHB-EGF by metalloproteinase" cannot serve as a compound which directly acts on a growth factor precursor in a G protein mediated extracellular signal transduction pathway which activates a growth factor receptor. Page 3, of the office action states that it is known that EGF is released from its precursor by metalloproteinase and EGFR is activated by EGF and thus ""CRM197 cannot serves "a compound which directly acts on a growth factor precursor in a G protein mediated extracellular signal transduction pathway which activates a growth factor receptor" as recited in claims 45 and 47"". Applicants respectfully point out that CRM197 directly binds to a growth factor precursor, namely Pro HB-EGF, which is part of a G protein mediated extracellular signal transduction pathway. The binding of CRM197 blocks the processing of the precursor by a metalloproteinase thereby interrupting the pathway and inhibiting the release of soluble growth factor which prevents the activation of a growth factor receptor. In view of this, applicants contend that CRM197 is a compound which directly acts on a growth factor precursor in a G protein mediated extracellular signal transduction pathway which activates a growth factor receptor and thus the claims are supported by the disclosure in the originally filed application.

The Examiner contends that the language "wherein said cancer cell is selected from the group consisting of pancreatic, prostate, gastric, breast, thyroid, pituitary, adrenal and ovarian tumor cells" in claims 44 and 45 is new matter with regard to *in vitro* methods. Applicants point out the disclosure on page 3, lines 23-27 of the present application which states that "the present invention provides methods for preventing or

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treating, among other diseases, hyperproliferative diseases such as colon, pancreatic, prostate, gastric, breast, lung, thyroid, pituitary, adrenal and ovarian tumors, as well as thyroid hyperplasia, retinitis pigmentosa, precocious puberty, acromegaly and asthma". In addition, pages 16 and 17 in the present application discuss human prostate cancer cells and page 6, lines 5-14 discuss the claimed method for identifying modulators of G-protein mediated signal transduction using a cell which contains a growth factor receptor. Since page 3 of the present application indicates that the present invention can be used to treat pancreatic, prostate, gastric, breast, thyroid, pituitary, adrenal and ovarian tumors, one skilled in the art would know that cells from these tumors have a growth factor receptor tyrosine kinase and thus one would reasonably expect such cells to be useful to identify test compounds as in claim 44. Regarding claim 45, applicants point out that treating the tumors discussed on page 3 of the present application using the present invention inherently results in the treatment of a cancer cell as in claim 45. In addition, page 4, lines 2-4 indicate that the contacting step may occur in vitro, e.g. in a cell culture or *in vivo*, e.g. in a subject in need of medical treatment. Therefore, the present inventors were in possession of and disclosed the subject matter claimed in claims 44 and 45. In view of the above discussion, applicants contend that the present claims do not include new matter and request that this rejection be withdrawn.

Claims 40-45, 47 and 48 are not vague and indefinite under 35 USC §112, second paragraph.

Claim 44 was rejected as vague and indefinite as to how one would know in which situation the test compound can be considered as a compound that has the ability to modulate a G-protein mediated signal transduction. Claim 44 includes the step of "evaluating G-protein mediated receptor tyrosine kinase activation upon exposure of the cancer cell to said test compound as an indication of said test compound's ability to modulate G-protein mediated signal transduction thereby identifying a test compound for modulating G-protein mediated signal transduction". One skilled in the art would know that if G-protein mediated receptor tyrosine kinase activation does not occur upon the exposure of the cancer cell to a test compound, then the test compound has modulated the G-protein mediated signal transduction. Claim 44 recites that the test compound is suspected to act on a precursor of a ligand of the receptor tyrosine kinase. If the test compound binds or acts on a precursor of a ligand of the receptor tyrosine kinase the G-protein mediated signal transduction pathway will be interrupted and there will not be G-protein mediated receptor tyrosine kinase activation. Applicants contend that one skilled in the art would know that if the test compound binds or acts on a precursor of a ligand of the receptor tyrosine kinase, there will be no G-protein mediated receptor tyrosine kinase activation. In view of the above discussion, applicants contend that claim 44 is not vague or indefinite.

Claims 45 and 47 were rejected as vague and indefinite regarding how to modulate the receptor tyrosine kinase activation by G-protein mediated signal

transduction. Claims 45 and 47 both recite stimulating G-protein mediated signal

transduction in a cell having a growth factor receptor tyrosine kinase and then

contacting the cell with a compound which directly binds to or acts on a growth

factor precursor in a G protein mediated extracellular signal transduction pathway

which activates a growth factor receptor. The compound binds to or acts on the

growth factor precursor, interrupting the signal transduction pathway and thereby

modulating the growth factor receptor tyrosine kinase activation by G-protein-

mediated signal transduction. Since both claim 45 and claim 47 recite that the

cell is contacted with a compound which directly binds to or acts on a growth

factor precursor in a G protein mediated extracellular signal transduction pathway

which activates a growth factor receptor, applicants contend that one skilled in

the art would know how the receptor tyrosine kinase activation is modulated by

G-protein mediated signal transduction.

Claims 44 and 45 are not obvious over Dong et al., Proc. Natl. Acad. Sci. USA, 96, 6235-6240 (May 1999) in view of Klemke et al., The Journal of Cell Biology, 127, 859-866 (1994) because claims 44 and 45 recite subject matter not shown or suggested by the cited prior art.

Klemke et al. was cited for the disclosure of a human pancreatic carcinoma cell

containing EGFR. Klemke does not suggest or disclose a method for modulating G-

protein modulated signal transduction. Dong et al. does not reference the G protein and

discusses only the inhibition of autocrine signal transduction by means of EGFR. Dong

teaches the incubation of HMEC cells with batimastat or mAb225 and then treating the

cells with EGF. Applicants note that on page 4 of the advisory action dated September

23, 2003, the examiner agrees that "one skilled in the art would not have expected

batimastat, which acts on an extracellular pathway of EGFR, to be capable of

modulating a G protein mediated signal transduction". Applicants respectfully point out

that at the time of the present invention, it was believed that the correlation between G

protein activation and the activation of tyrosine phosphorylation of EGFR was mediated

by an intracellular pathway. Thus, one skilled in the art would not have expected

batimastat, which acts on an extracellular pathway of EGFR, to be capable of

modulating a G protein mediated signal transduction. As indicated on page 6 of the

office action dated May 20, 2003, Dong does not directly show that their method is

related to modulation of G-protein mediated signal transduction. Applicants contend

that in view of the knowledge in the art, one skilled in the art would not have been

motivated to modify Dong's method to modulate G protein mediated signal transduction.

In contrast to the advisory action dated September 23, 2003, the office action

dated May 20, 2003 states: "*since it is known that reduction of tyrosine phosphorylation*

of a receptor is correlated to activation of G protein, batimastat used in the method of

Dong et al. also modulate G protein mediated signal transduction." Applicants point out

that this statement is both incomplete and incorrect. A correlation of G protein

activation and the activation of tyrosine phosphorylation of EGFR was indeed known in

the art. It was assumed, however, that this correlation is mediated by an intracellular

pathway. Thus, prior to the present invention, one skilled in the art could not reasonably

have expected that batimastat as used in the method of Dong et al. (which acts on a

different extracellular activation pathway of EGFR mediated by addition of EGF) would

be capable of modulating a G protein mediated signal transduction. The activation of

receptor tyrosine kinases such EGFR can be effected via a plurality of different

pathways. As explained in detail below, a number of different stimuli were known, in

addition to the activation of G proteins, which were correlated with EGFR tyrosine

phosphorylation at the time of the Dong et al publication. For example, it had been

reported at the time of Dong et al. that the stimulation of cytokine receptors leads to

tyrosine phosphorylation of the EGF receptor (Yamaguchi et al., 1997, Tyrosine

phosphorylation of the EGF receptor by the kinase JAK2 is induced by growth hormone.

Nature 390: 91-96). However, in contrast to the mechanism observed by Dong et al.,

this pathway involves the JAK family of intracellular non-receptor tyrosine kinases and

requires the intracellular adaptor-docking function of the EGF receptor (Yamaguchi et

al., 1997).

It was also known at the time of Dong et al. that members of the integrin family of

cell surface receptors can modulate EGFR tyrosine phosphorylation in order to generate

further cellular responses (Moro et al., 1998, Integrins induced activation of the EGF

receptor: role in MAP kinase induced and adhesion-dependent cell survival. EMBO J

17: 6622-6632). However, again in contrast to the mechanism observed by Dong et al.,

this pathway is independent of EGFR ligands and involves cell adhesion dependent

interaction of integrins with the EGFR (Moro et al., 1998).

Furthermore, it was well documented at the time of Dong et al. that a number of

exogenous stress stimuli, both physical and chemical, initiate signal transduction

pathways in cells that are part of stress responses. In particular, UV radiation had been

reported to promote enhanced tyrosine phosphorylation of the EGF receptor (Warmuth

et al., 1994. Ultraviolet radiation induces phosphorylation of the epidermal growth factor receptor. Cancer Res. 54: 374-376). However, as UV activates v-erbB, an oncogenic isoform of the chicken EGF-receptor that lacks a ligand-binding domain, the mechanism of UV-induced EGFR activation occurs ligand-independently (Knebel et al 1996, Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents. EMBO J. 15: 5314-5325). In fact, activation of the EGFR is indirect through inactivation of phosphotyrosine phosphatases (Knebel et al., 1996). Finally, it had been demonstrated at the time of Dong et al. that the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) promotes EGFR tyrosine phosphorylation via its intracellular receptor protein kinase C (Xian, W. et a., 1995, Activation of the epidermal growth factor receptor by skin tumor promoters and in skin tumor from SENCAR mice, Cell Growth & Differentiation 6: 1447-1455; Emkey and Kahn, 1997, Cross-talk between phorbol ester-mediated signaling and tyrosine proto-oncogenes, J. Bio. Chem. 272: 31172-31181). The present application (see also Prenzel et al., 1999) discloses that this TPA-induced transactivation of the EGF receptor occurs via metalloproteases and HB-EGF but is clearly distinct from GPCR mediated EGFR receptor activation since the receptor activation is not by GPCR (see Figures 2c and 3a of the present application).

Applicants contend that one skilled in the art would not assume from the mere correlation of the effect of the compound according to Dong et al. on the proteolytic release of EGFR ligands that it modulates GPCR mediated EGFR tyrosine phosphorylation, particularly since there were non-GPCR mediated EGFR tyrosine

phosphorylation pathways which are also inhibited by batimastat. At the time of Dong et al. it was commonly believed that GPCR stimulation of EGFR activation itself did not involve proteolytic cleavage of ligand precursors. For example, at the time of Dong et al. it was generally acknowledged that the mechanism by which GPCRs modulate tyrosine phosphorylation of the EGF receptor is centered on the mediation by the non-receptor tyrosine kinase c-Src, which was reported to be coupled to nearly all GPCRs that lead to EGF receptor phosphorylation (reviewed in Thomas and Brugge, 1997, Cellular functions regulated by Src family kinases. Annu. Cell Dev. Biol. 13: 513-609). Over expression of either a dominant-negative Src construct or Csk, a regulatory kinase that inhibits Src function, decreases EGF receptor tyrosine phosphorylation provoked by activation of LPA or α2 adrenergic receptors (Luttrell et al., 1997, Gby subunits mediated Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor mediated Ras activation. J. Biol. Chem. 272:4637-4644). The mediator role of Src was suggested to be direct in that Src is able to associate with and phosphorylate the EGF receptor *in vivo* and *in vitro* (Thomas and Brugge, 1997). This mechanism would predict the existence of Src-EGF receptor complexes provoked by activation of GPCR. The evidence for this was shown by the demonstrations that angiotensin II (Eguchi et al., 1998, Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular 2smooth muscle cells, J. Biol. Chem. 273: 8890-8896) or LPA (Luttrell et al., 1997) rapidly increases the amount of Src coprecipitated with EGF receptors.

Furthermore, calcium influx was reported to be sufficient to trigger EGFR tyrosine kinase phosphorylation and MAP kinase activation in PC12 cells (Rosen and Greenberg, 1996, Stimulation of growth factor receptor signal transduction by activation of voltage-sensitive calcium channels. Proc. Natl. Acd. Sci. USA 93: 1113-1118). This had been extended subsequently since several findings had demonstrated Ca^{2+} to be necessary for EGFR-transactivation induced by GPCR-ligands (Eguchi et al., 1998; Eguchi et al., 1999, Involvement of PYK2 in angiotensin II signaling of vascular smooth muscle cells, Hypertension 33: 201-206; Soltoff 1998, Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled P-2Y2 receptor, J. Biol. Chem 273: 23110-23117). Due to this critical function of Ca^{2+}, Ca-regulated FAK family kinase PYK2 was discussed as a mediator of EGFR transactivation in the signaling elicited by GPCR ligands upstream of the EGFR signal (Eguchi, 1999; Soltoff 1998).

As discussed above, the correlation of GPCR-induction of EGFR activation via a pathway comprising extracellular elements is not suggested or disclosed by Dong et al. Thus one skilled in the art would not be motivated to modify Dong et al to contact a cancer cell with a compound which affects an extracellular signal pathway as required by claims 44 and 45.

Enclosed is a diagram which shows the signal transduction pathway starting from a dysfunction of G-protein signal transduction and resulting in a receptor tyrosine kinase activation. In the presently claimed method, the modulator acts on a precursor of a ligand of the receptor tyrosine kinase or on a growth factor precursor in a G-protein

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mediated extracellular signal pathway which activates a growth factor receptor. In contrast to claims 44 and 45, Dong uses batimastat which inhibits the metallo-proteinase. Thus, the present invention inhibits receptor tyrosine kinase transactivation by a different mechanism. For example, the recognition sequence for a metalloproteinase may be masked by binding of the modulator in the present method or the binding of the modulator may inhibit the binding of the growth factor to the receptor. Though Dong's method can interrupt the whole signal cascade, the present invention interrupts the signal cascade in a different way. In the present invention a compound acts on the growth factor precursor inhibiting processing of the precursor and interrupting the signal cascade. Thus, the present inventors have shown for the first time that modulators which act on a growth factor precursor to inhibit the activation of the extracellular domain of a growth factor receptor are suitable for the treatment of disorders, in particular of cancers, which are induced by G- protein mediated signal transduction. In contrast to the present invention, Dong discloses only that the inhibitory effect of batimastat on metastasis is due to interference with autocrine EGFR signaling. Thus, Dong does not suggest or disclose a method for identifying and providing modulators according to the present invention as Dong suggests only Batimastat, which is a compound which acts on a metalloproteinase. In addition, applicants respectfully point out that the addition of EGF as disclosed in Dong, does not activate the G-protein or GPCR initiated extracellular signal transduction pathway. The addition of EGF causes a stimulation of EGFR which is different from the stimulation which proceeds via the G-protein or GPCR initiated extracellular signal transduction pathway as

required in the present claims. Figure 4 in the present application shows the influence of the addition of various reagents such as TPA, bombesin, carbachol and EGF, on the stimulation of the GPCR-initiated signal transduction pathway. This stimulation is shown by the detection of processed HB-EGF-1 (Fig 4A). Though the detection of processed HB-EGF-1 is possible after the addition of TPA, bombesin, and carbachol, no HB-EGF-1 is detectable after preincubation with EGF (Fig. 4A, right column). Figure 4a in the present application clearly shows that EGF, in contrast to TPA, bombesin and carbachol, is not an activator of the GPCR initiated signal transduction pathway. In view of this, applicants contend that Dong does not suggest stimulating G-protein mediated signal transduction in a cancer cell or contacting the cell with a compound which acts on a growth factor precursor as required in claims 44 and 45.

In summary, Dong and Klemke individually and in combination do not reference the G protein and Dong only discusses the inhibition of autocrine signal transduction by means of EGFR, Dong uses batimastat which inhibits the metallo-proteinase and at the time of the present invention, it was believed that the correlation between G protein activation and the activation of tyrosine phosphorylation of EGFR was mediated by an intracellular pathway while batimastat acts on an extracellular pathway of EGFR. Therefore, one skilled in the art would not have modified Dong's method in view of Klemke and in order to modulate a G protein mediated signal transduction in a cancer cell. Therefore, the applicant submits that the outstanding rejection of claims 44 and 45 is improper and should be withdrawn.

Conclusion

For all of the above noted reasons, it is strongly contended that claims 40-43, 45, 47 and 48 comply with the written description requirement under 35 USC §112, claims 40-45, 47 and 48 are not vague and indefinite under 35 USC §112, second paragraph and that certain clear differences exist between the present invention as claimed in claims 44 and 45 and the prior art relied upon by the Examiner. It is further contended that these differences are more than sufficient evidence that the present invention would not have been obvious to a person having ordinary skill in the art at the time the invention was made.

This final rejection being in error, therefore, it is respectfully requested that this honorable Board of Patent Appeals and Interferences reverse the Examiner's decision in this case and indicate the allowability of claims 40-45, 47 and 48.

In the event that this paper is not being timely filed, the Patent Owner respectfully petitions for an appropriate extension of time. Please charge any fee or credit any overpayment pursuant to 37 §C.F.R. 1.16 or §1.17 to Deposit Account No. 02-2135.

Respectfully submitted,

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VIII.

APPENDIX OF CLAIMS ON APPEAL

Claims 1-39 (Cancelled)

40. The method according to claim 47, wherein said receptor tyrosine kinase is epidermal growth factor receptor (EGFR).

41. The method according to claim 47, wherein said growth factor precursor is proheparin-epidermal growth factor (proHB-EGF) and said receptor tyrosine kinase is EGFR.

42. The method according to claim 47, wherein said receptor tyrosine kinase is selected from the group consisting of epidermal growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER-2), human epidermal growth factor receptor-3 (HER-3), human epidermal growth factor receptor-4 (HER-4), Tumor Necrosis Factor receptor 1 (TNF receptor 1), Tumor Necrosis Factor receptor 2 (TNF receptor 2), tumor necrosis factor receptor superfamily, member 8 (CD 30) and interleukin 6 receptor (IL-6 receptor).

43. The method according to claim 47, wherein said receptor tyrosine kinase is selected from the group consisting of EGFR and other members of the EGFR family.

44. A method for identifying a test compound for modulating G-protein mediated signal

transduction, comprising contacting a cancer cell containing a receptor tyrosine kinase

capable of activation by G-protein mediated signal transduction with a test compound

suspected to act on a precursor of a ligand of the receptor tyrosine kinase, and

evaluating G-protein mediated receptor tyrosine kinase activation upon exposure of the

cancer cell to said test compound as an indication of said test compound's ability to

modulate G-protein mediated signal transduction thereby identifying a test compound

for modulating G-protein mediated signal transduction, wherein said cancer cell is

selected from the group consisting of pancreatic, prostate, gastric, breast, thyroid,

pituitary, adrenal and ovarian tumor cells.

45. A method for modulating growth factor receptor activation by modulating a G-protein

mediated signal transduction, comprising:

stimulating G protein mediated signal transduction in a cancer cell having a

growth factor receptor tyrosine kinase, wherein the growth factor receptor tyrosine

kinase is activated, and wherein said growth factor receptor tyrosine kinase is selected

from the group consisting of EGFR and other members of the EGFR family, said cancer

cell comprising an extracellular EGFR domain and having a G-protein mediated signal

transduction pathway which activates a growth factor receptor, wherein one or more

tyrosine residues are phosphorylated based on the activation of said G-protein

mediated signal transduction pathway, the extracellular domain of said receptor is

capable of binding to its receptor ligand, and said ligand is generated from a precursor of said ligand by a proteinase-dependent cleavage; and

contacting said cancer cell with a compound which acts on a growth factor precursor in a G protein mediated extracellular signal pathway which activates a growth factor receptor, and thereby modulating the growth factor receptor tyrosine kinase activation by G-protein mediated signal transduction, wherein said cancer cell is selected from the group consisting of pancreatic, prostate, gastric, breast, thyroid, pituitary, adrenal and ovarian tumor cells.

46. (Canceled)

47. A method for modulating growth factor receptor activation by modulating G-protein mediated signal transduction comprising:

stimulating G protein mediated signal transduction in a cell having a growth factor receptor tyrosine kinase, wherein the growth factor receptor tyrosine kinase is activated; and

contacting the cell with a compound which directly binds to a growth factor precursor in a G protein mediated extracellular signal transduction pathway which activates a growth factor receptor, wherein said G protein mediated extracellular signal transduction pathway includes cleavage of a growth factor precursor, thereby modulating the growth factor receptor tyrosine kinase activation by G-protein-mediated signal transduction.

48. The method according to claim 47, wherein said cell is an ovarian cancer cell or a

prostate cancer cell.

IX.

Evidence Appendix

A copy of the background references discussed above and in the applicant's prior responses are included with this brief. These references were discussed in applicant's August 20, 2003 response. Also included is a chart showing the G-protein signal transduction pathway which was submitted with applicant's November 28, 2005 response.
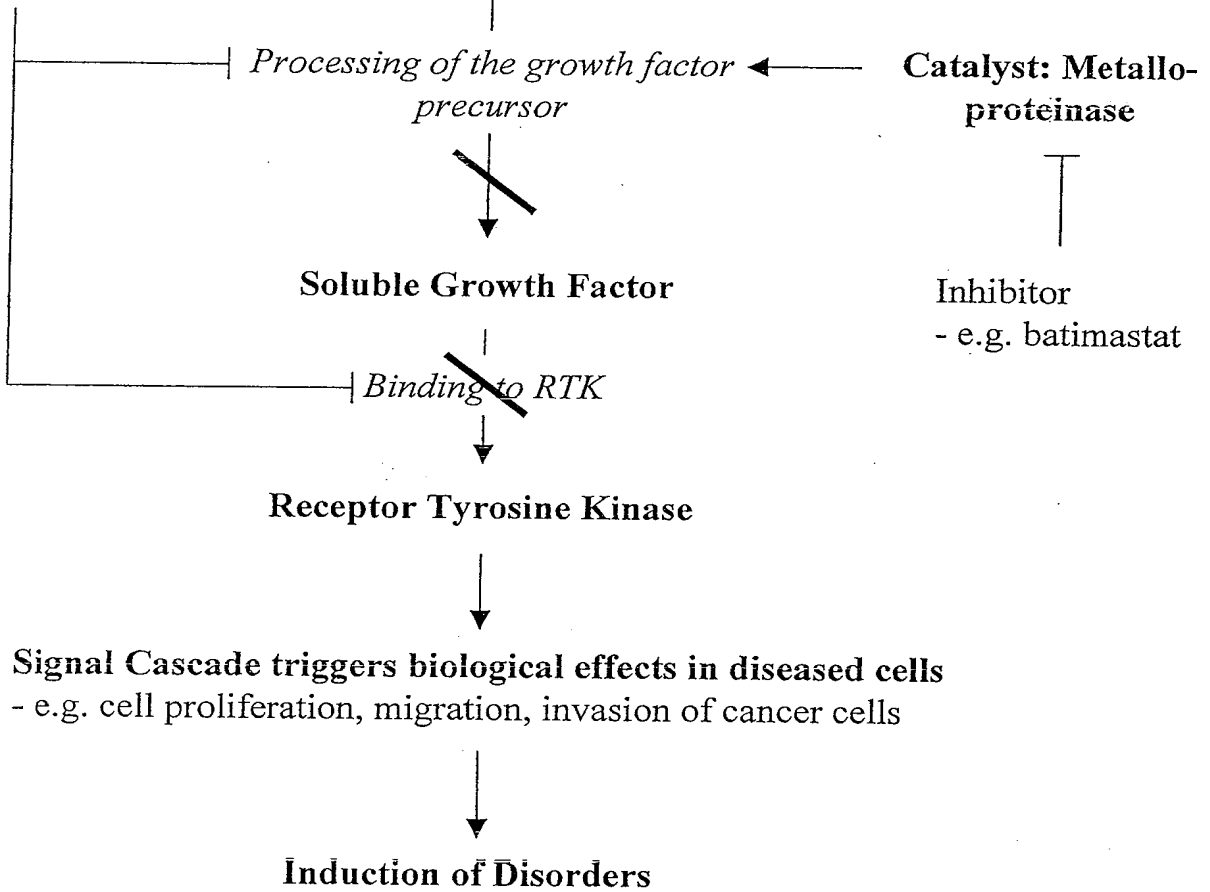
X.

RELATED PROCEEDINGS APPENDIX

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Dysfunction of G-protein signal transduction

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Stimulation via G-Protein/GPCR

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**Modulator of
    Claim1** — *binding* → **Growth Factor precursor**
-e.g. CRM197

|
Processing of the growth factor ◄——— **Catalyst: Metallo-
    precursor**   **proteinase**

⊤

↓

Soluble Growth Factor   Inhibitor
                                                            - e.g. batimastat

|
Binding to RTK

↓

Receptor Tyrosine Kinase

|
↓

Signal Cascade triggers biological effects in diseased cells
- e.g. cell proliferation, migration, invasion of cancer cells

|
↓

Induction of Disorders

enhanced coupling efficiency to G_i results in direct feedback inhibition of the G_s-mediated adenylyl cyclase signal that initiated the process. Moreover, coupling of the β-AR to G_i initiates a second wave of G_i-mediated signalling, including the rapid activation of MAP kinases, which coincides with termination of the signal that initiated the original response. Thus, PKA-mediated phosphorylation of the β-AR serves as a switching mechanism to regulate the G-protein-coupling specificity of the receptor. □

Methods

Cell culture and transfection. The cDNAs encoding wild-type β_2-AR, β_2-AR lacking putative phosphorylation sites for cAMP-dependent protein kinase A (β_2-AR$_{mut}$; point mutations of serine residues at 261, 262, 345 and 346 to alanine), the β-ARK C-terminal peptide-encoding minigene (β-ARKct) and the C-terminal fragment of mSos1 (Sos-Pro) were constructed in our laboratory[5,9,15]. The cDNAs encoding p50^{csk} and haemagglutanin-tagged p44^{mapk} (p44$^{HA-mapk}$) were provided by H. Hanafusa and J. Pouysségur, respectively. Cells were collected and re-established in fresh medium 24 h before each experiment. Transient transfections were done using calcium phosphate co-precipitation[27]. Cells were starved overnight in medium containing 10 mM HEPES (pH 7.4) and 0.1% (v/v) BSA before agonist stimulation. All assays were done 48 h after transfection. Transient expression of plasmids transfected with p50^{csk}, mutant mSos1 or β-ARKct was verified by immunoblotting of whole-cell lysates using commercially available antibodies.

Phospho-MAPK assay. HEK293 cells were treated with isoprenaline (1 μM) or lysophosphatidic acid (10 μM) for 5 min at 37 °C. Cell monolayers were lysed in Laemmli sample buffer and lysates resolved by SDS–PAGE. Activated MAP kinase on nitrocellulose was detected using anti-phospho-MAP-kinase antibodies (New England BioLabs), visualized by enzyme-linked chemiluminescence (Amersham) and quantified by scanning laser densitometry.

Gi loading. Membranes[28] from HEK293 cells were aliquoted and stored at −70 °C in TE (20 mM Tris, pH 7.4, 1 mM EDTA) buffer. Membrane proteins (40–50 μg) were preincubated in TE buffer containing 50 mM CH$_3$CO$_2$Na, 0.2 mM EGTA, 1.0 mM benzamidine, 2 mM MgCl$_2$ and 50 μM GDP in order to load Gα subunits. Isoprenaline and [γ-^{32}P]GTP-azidoanilide (Research Products) were added and samples incubated for 10 min at ambient temperature. The reaction was terminated by centrifugation (14,000g for 10 min) and membrane pellets were resuspended in TE containing 1 mM dithiothreitol and irradiated for 10 min with an ultraviolet lamp (λ = 254 nm). Membranes were collected by centrifugation and solubilized in 40 μl of 2% SDS followed by the addition of 800 μl of RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 0.5% deoxycholate, 10 mM NaF, 1 mM Na$_3$VO$_4$, 0.2 mM PMSF and 10 μg aprotinin per ml). Samples were incubated with 15 μg anti-G$_i$ antiserum and 50 μl protein A/G–Sepharose beads and rotated overnight at 4 °C. Beads were washed three times with ice-cold RIPA buffer, resuspended in Laemmli buffer and separated by SDS–PAGE. Proteins were transferred onto nitrocellulose filter and analysed for [γ-^{32}P]GTP incorporation by autoradiography. Filters were also analysed for immunoprecipitation efficiency by blotting for the presence of G$_i$ proteins.

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Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone

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When growth hormone binds to its receptor, which belongs to the cytokine receptor superfamily[1], it activates the Janus kinase Jak2[2] which has tyrosine-kinase activity and initiates an activation of several key intracellular proteins (for example, mitogen-activated protein (MAP) kinases[3–6]) that eventually execute the biological

actions induced by growth hormone, including the expression of particular genes. In contrast to receptors that themselves have tyrosine kinase activity, the signalling pathways leading to MAP kinase activation[7,8] that are triggered by growth hormone are poorly understood, but appear to be mediated by the proteins Grb2 and Shc[9]. We now show that growth hormone stimulates tyrosine phosphorylation of the receptor for epidermal growth factor (EGFR) and its association with Grb2 and at the same time stimulates MAP kinase activity in liver, an important target tissue of growth hormone. Expression of EGFR and its mutants revealed that growth-hormone-induced activation of MAP kinase and expression of the transcription factor c-fos requires phosphorylation of tyrosines on EGFR, but not its own intrinsic tyrosine-kinase activity. Moreover, tyrosine at residue 1,068 of the EGFR is proposed to be one of the principal phosphorylation sites and Grb2-binding sites stimulated by growth hormone via Jak2. Our results indicate that the role of EGFR in signalling by growth hormone is to be phosphorylated by Jak2, thereby providing docking sites for Grb2 and activating MAP kinases and gene expression, independently of the intrinsic tyrosine kinase activity of EGFR. This may represent a novel cross-talk pathway between the cytokine receptor superfamily and growth factor receptor.

Insulin-receptor substrates IRS-1 and IRS-2 are tyrosine-phosphorylated in response to growth hormone (GH) in cultured cells such as primary rat adipocytes, 3T3-F442A fibroblasts, and CHO-GH receptor (GHR) cells[10-12]. We detected GH-dependent tyrosyl phosphorylation of a protein with a relative molecular mass (M_r) of 180K (pp180), Jak2 and GHR (Fig. 1a, top, lane 3, and data not shown) in mouse liver, a major target tissue, in vivo: its maximal

tyrosine phosphorylation was roughly equivalent to that of IRS-1 (M_r 170K) observed following treatment with insulin (Fig. 1a, top, lane 2). IRS-1 deficiency[13-15] had no apparent effect on GH-induced tyrosine phosphorylation of pp180 (Fig. 1a, top, lane 6). In addition, although IRS-1 deficiency induced tyrosine phosphorylation of IRS-2 (M_r ~180K) instead of IRS-1 in response to insulin (Fig. 1a, top, lane 5), immunodepletion of IRS-2 using specific antibody did not cause a significant decrease in tyrosine phosphorylation of pp180 in response to GH (data not shown). Thus the identity of the pp180, the principal protein tyrosine-phosphorylated in response to GH, is not IRS-1/IRS-2. GH stimulated association of pp180 with Grb2 but not with p85 phosphatidylinositol-3-OH kinase (PI(3)K) (data not shown). In addition, we were unable to extract pp180 without detergent, and found that it bound to wheat germ agglutinin-linked beads. We therefore tested whether EGFR could be pp180 by stripping and reprobing the sheet in Fig. 1a (top) with anti-EGFR antibody. We found that GH stimulated tyrosine phosphorylation of EGFR, whereas insulin did not (Fig. 1a, bottom). Moreover, immunodepletion of EGFR using specific antibody revealed that pp180 was almost entirely EGFR (Fig. 1b).

EGFR was tyrosine-phosphorylated in a time- and dose-dependent manner in response to GH in liver in vivo (Fig. 1c, d). In addition, EGFR was associated with Grb2 in response to GH (Fig. 1e). Shc was also tyrosine-phosphorylated in response to GH (Fig. 1f, lane 3). These results indicated that EGFR and Shc may represent two different pathways by which GH can activate MAP kinase (Fig. 1g), presumably through Grb2. Using specific antibodies, we found that GH could also induce tyrosine phosphorylation of IRS-1/IRS-2 in liver, albeit much less efficiently than insulin or the tyrosine
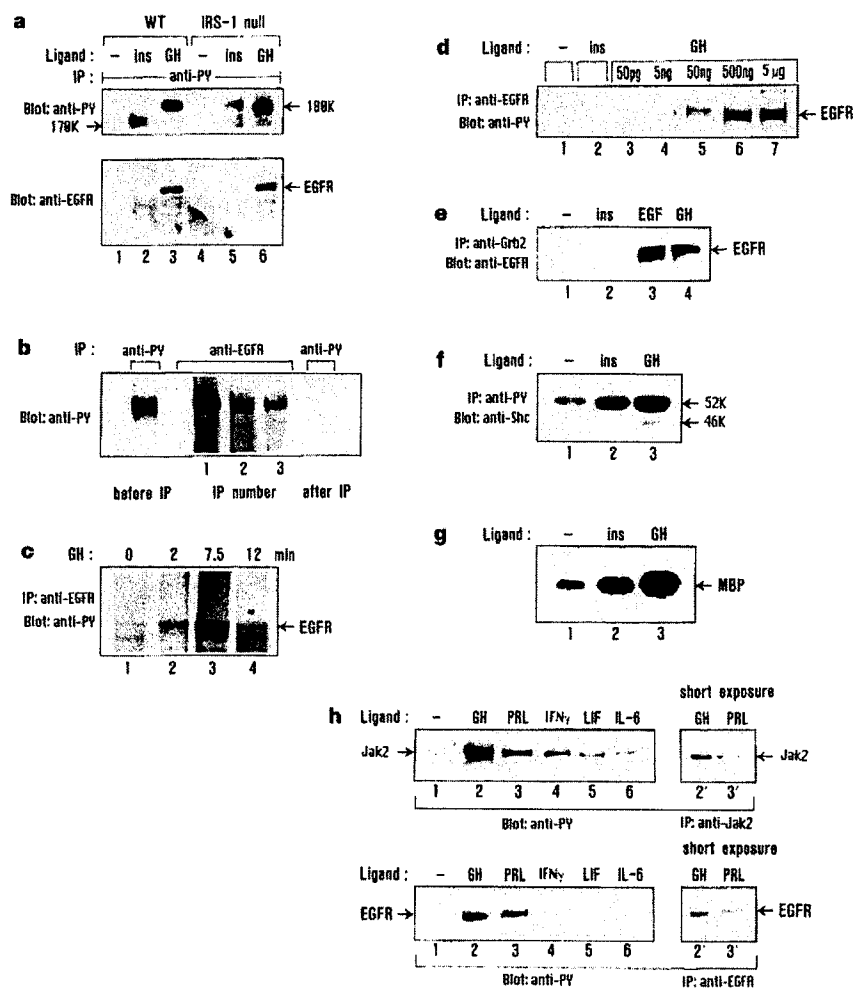

Figure 1 GH-stimulated tyrosine phosphorylation of EGFR and its association with Grb2, and concomitantly stimulated MAP kinase activity in liver in vivo. Wild-type (WT) or IRS-1-deficient mice (IRS-1 null) were treated with or without 1 U g^{-1} body weight (BW) of insulin (ins) or 500 ng g^{-1} BW of EGF for 5 min or 5 μg g^{-1} BW of hGH or PRL or 1 μg g^{-1} BW of IFN-γ or IL-6 or 2.5 μg g^{-1} BW of LIF for 7.5 min (a, e, f, g, h) or treated with 5 μg g^{-1} BW of hGH for 7.5 min for the indicated time periods (c) or the indicated doses of hGH for 7.5 min (d). Liver lysates were immunoprecipitated (IP) with anti-PY (a, f) or anti-EGFR (c, d, h, bottom) or anti-Grb2 (e) or anti-Jak2 (h, top), followed by immunoblotting with anti-PY (a, top, c, d, h) or anti-EGFR (a, bottom, e) or anti-Shc (f). When bovine or human GH was injected, we obtained similar results (data not shown). b, Immunodepletion with anti-EGFR (see Methods). g, Liver extracts were immunoprecipitated with anti-MAP kinase (αC92; ref. 15), followed by kinase assay towards myelin basic protein (MBP). Labelled MBP was subjected to SDS-PAGE and autoradiography.

phosphorylation of EGFR stimulated with GH (unpublished observations).

We next tested whether other cytokine family members that activate Jak2 and have their own receptors in liver can stimulate tyrosine phosphorylation of EGFR. Interleukin-6 (IL-6), leukaemia inhibitory factor (LIF) and interferon-γ (IFN-γ) were able to activate Jak2 to 10–30% of the extent of GH and prolactin. However, only GH and prolactin, not IL-6, LIF or IFN-γ could stimulate tyrosine phosphorylation of EGFR (Fig. 1h), suggesting that it is specifically induced by GH and prolactin.

To study the role of tyrosine phosphorylation of EGFR in GH signalling, we used CHO cells stably transfected with the human GHR complementary DNA (CHO-GHR cells), which do not express endogenous EGFR. Wild-type (WT) EGFR or LacZ as a control was introduced into the cell line by infection with a recombinant adenovirus[16]. In CHO-GHR cells expressing LacZ, GH failed to induce tyrosine phosphorylation of EGFR (Fig. 2a, lane 3). In contrast, in CHO-GHR cells expressing WT EGFR, GH induced tyrosine phosphorylation of EGFR in a dose-dependent manner (Fig. 2a lane 6, and data not shown). Expression of WT EGFR in these cells was accompanied by GH-induced MAP kinase activation, significantly enhanced compared with cells expressing no EGFR at all the time points and GH concentrations studied (Fig. 2b, and data not shown). The association of tyrosine-phosphorylated proteins with Grb2 is a crucial step in the activation of the MAP-kinase cascade. Stimulation of CHO-GHR cells expressing LacZ by GH caused a 52K tyrosine-phosphorylated protein (Shc) to associate with a glutathione-S-transferase (GST)–Grb2 fusion protein (Fig. 2c, lane 2). In contrast, expression of WT EGFR resulted in GH-dependent association of EGFR with Grb2, which concomitantly reduced Shc binding to Grb2 (Fig. 2c,

lane 4), suggesting that there may be competition between EGFR and Shc. We next examined another cell line which expresses extremely low amounts of endogenous EGFR, INS-1 (ref. 17) (Fig. 2d, bottom). Stimulation of MAP kinase by GH was defective in INS-1 (Fig. 2e, lane 3), and could be restored by expression of EGFR (Fig. 2e, lane 6) through GH-induced tyrosine phosphorylation of EGFR (Fig. 2d, top, lane 6) and its association with Grb2 (data not shown).

To study the mechanisms of GH-induced tyrosine phosphorylation of EGFR, kinase-negative EGFR or an EGFR mutant with its main tyrosine-autophosphorylation sites substituted by phenylalanine (F5-EGFR) was introduced into CHO-GHR cells. In cells expressing kinase-negative EGFR, GH induced tyrosine phosphorylation of EGFR, which became associated with Grb2, and comcomitantly stimulated MAP-kinase activity to an extent comparable to cells expressing WT EGFR (Fig. 3a, b, lanes 4, 6). In contrast, in CHO-GHR cells expressing F5-EGFR, there was little tyrosine phosphorylation of EGFR or association with Grb2 (Fig. 3a, lane 8), and GH stimulated MAP-kinase activity only to levels seen in cells expressing no EGFR (Fig. 3b, lanes 2, 8). These results indicate that full GH-induced activation of MAP kinase required tyrosine-phosphorylation sites on EGFR but did not depend on its intrinsic tyrosine-kinase activity. Tyrosine at residue 1,068 of EGFR is a major binding site for Grb2, when stimulated with EGF[18,19]. A quadruple point mutant retaining one tyrosine at position 1,068 out of five tyrosine-autophosphorylation sites (F4/Y1068) was tyrosine-phosphorylated and could recruit Grb2 and induce activation of MAP kinase to almost the same extent as WT EGFR in response to GH in CHO-GHR cells (Fig. 3a, b, lanes 4, 10). This essential role of tyrosine-phosphorylation of EGFR was confirmed by the observation that overexpression of F5-EGFR (Fig. 3c, lanes 5, 6) significantly
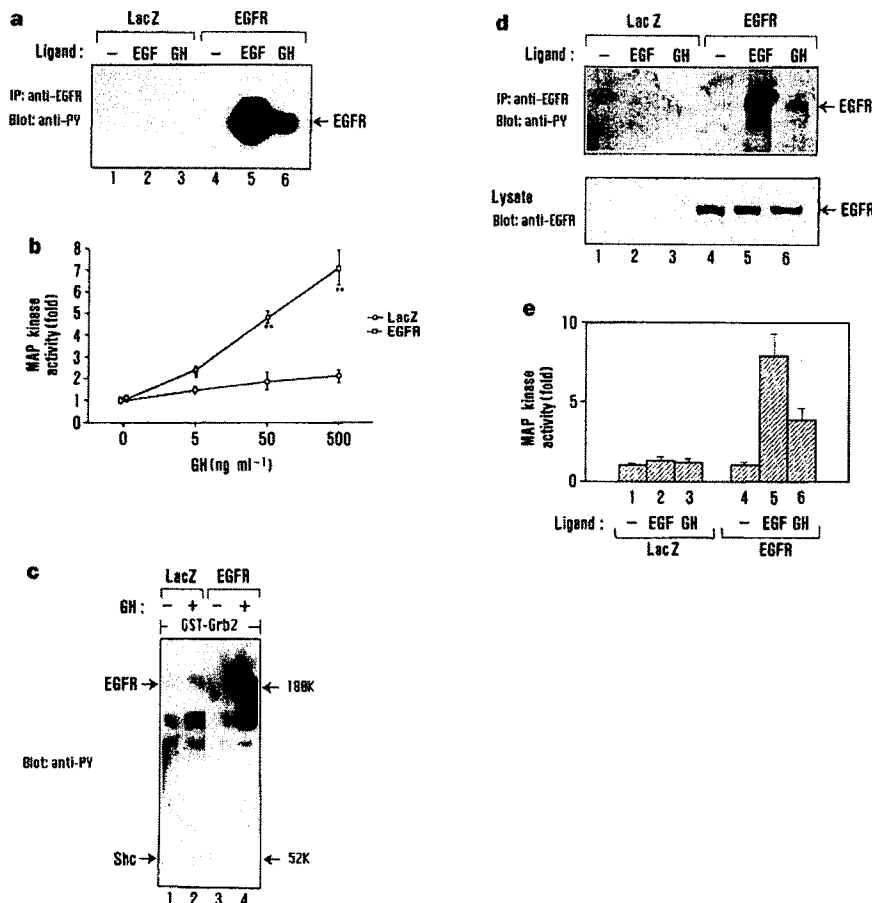

Figure 2 Tyrosine phosphorylation of EGFR enhances GH-induced MAP-kinase activation. a, c, d, Quiescent CHO-GHR cells (a, c) or INS-1(d) expressing wild-type EGFR or LacZ as a result of adenovirus-mediated gene transfer were stimulated for 5 min with EGF (100 ng ml⁻¹) or hGH (500 ng ml⁻¹). Upon lysis, precipitates with anti-EGFR (a, d, top) or GST–Grb2 (c) were immunoblotted with anti-PY (a, c, d, top). Equal expression was confirmed by probing with an anti-EGFR antibody in whole-cell lysates (d, bottom). b, e, Immune complex MAP-kinase assay. Quiescent CHO-GHR cells (b) or INS-1 (e) were stimulated with the indicated doses of hGH for 10 min (b) or with EGF (100 ng ml⁻¹) or hGH (500 ng ml⁻¹) for 10 min (e), and MAP kinase activity was determined as described[15]. Results are expressed as the ratio to the value of untreated CHO-GHR cells expressing LacZ. Each bar represents the mean ± s.e. of more than three independent experiments (*P < 0.01; **P < 0.001; LacZ versus EGFR).
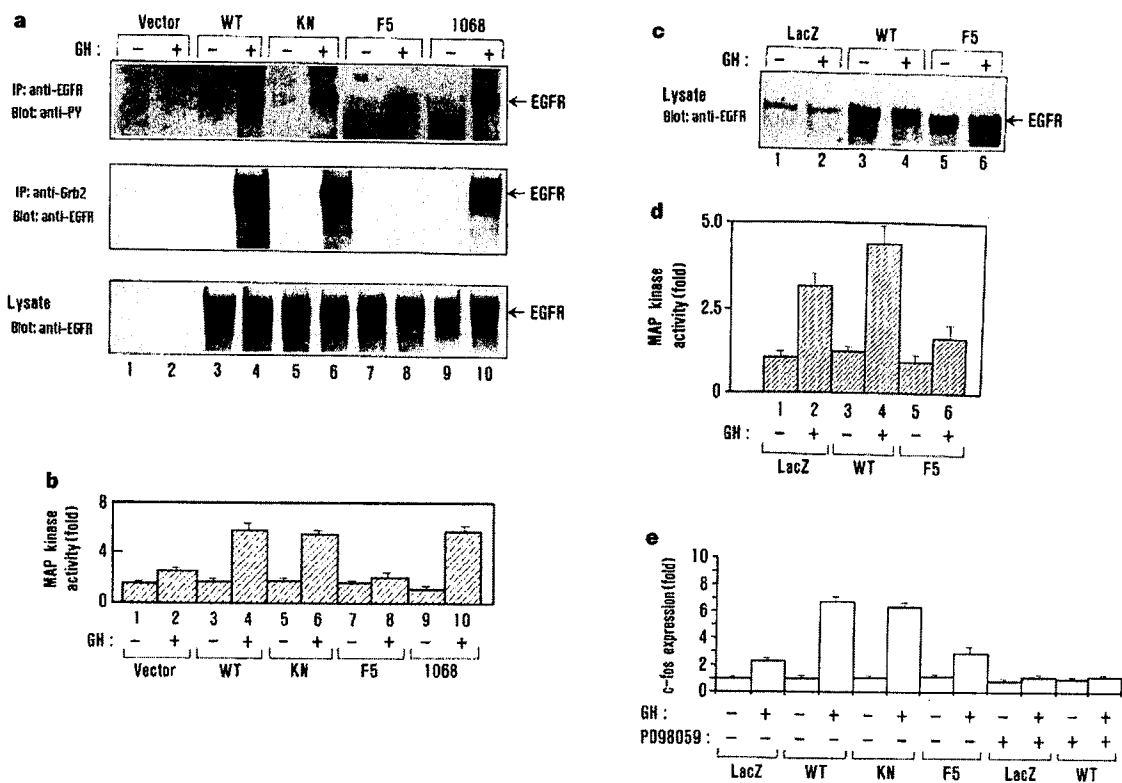
Figure 3 GH-induced full activation of MAP kinase and gene expression requires tyrosine-phosphorylation sites of EGFR but not EGFR intrinsic kinase activity. a, b, Quiescent CHO-GHR cells transfected with WT or EGFR mutant plasmids were stimulated with hGH (500 ng ml⁻¹) for 5 min (a) or for 10 min (b). Upon cell lysis, precipitated EGFR was immunoblotted with anti-PY (top), and immunoprecipitates by anti-Grb2 were immunoblotted with anti-EGFR (centre). Equal expression was confirmed by probing with anti-EGFR in whole-cell lysates (bottom) (a). MAP-kinase activity was determined by immune-complex assay as described[15]. Results are expressed as the ratio to the value of untreated CHO-GHR cells expressing no EGFR. Representative data of one of three independent experi-

ments are shown; values are means ± s.d. (b). c, Expression of WT or F5-EGFR in 3T3 F442A cells via adenovirus-mediated gene transfer was confirmed by probing with anti-EGFR in whole-cell lysates. d, Immune complex MAP-kinase assay. Quiescent 3T3 F442A cells were stimulated for 10 min with hGH (500 ng ml⁻¹) and processed as in Fig. 2. e, GH-induced c-fos expression in CHO-GHR cells expressing LacZ or WT or kinase-negative (KN) or F5-EGFR with or without pretreatment with 30 μM PD98059 for 60 min was determined as in Methods. Results are expressed as the ratio to the value of c-fos expression in untreated CHO-GHR cells expressing LacZ. Representative data from one of three independent experiments are shown; values are means ± s.d.

reduced GH-stimulated MAP-kinase activation in 3T3 F442A cells (Fig. 3d, lanes 2, 6), which express endogenous EGFR (Fig. 3c, lanes 1, 2).

To determine the importance of GH-induced tyrosine phosphorylation of EGFR in the function of GH, we studied GH-induced expression of c-fos. Full induction of c-fos by GH requires several regulatory elements in the promoter, including the c-sis-inducible element known as STAT (signal transducers and activators of transcription) 1 and 3-binding element and the ternary complex factor (TCF) element[20]. Maximal transcriptional activity of both STAT proteins[21] and TCF[22] requires MAP kinases. As expected, in CHO-GHR cells expressing WT or kinase-negative EGFR, GH significantly stimulated c-fos expression compared with cells expressing LacZ or F5-EGFR, and this GH-induced increase in c-fos expression was almost abolished by pretreatment of cells with the MAP kinase kinase inhibitor PD98059 (Fig. 3e).

We next examined whether GH-induced tyrosine phosphorylation of EGFR is dependent on Jak2 by using γ2A/GHR[5] cells, which lack Jak2. GH was unable to induce tyrosine phosphorylation of EGFR (Fig. 4a, lane 3) nor to stimulate MAP-kinase activation of γ2A/GHR cells transiently transfected with WT EGFR (data not shown), whereas complementation with a Jak2 expression plasmid restored these responses (Fig. 4a, lane 6, and data not shown). Furthermore, expression of ΔJak2, which inhibits autophosphorylation of WT JAK2 in a dominant-negative manner[23], largely reduced GH-induced tyrosine phosphorylation of EGFR in CHO-

GHR transiently transfected with WT EGFR (Fig. 4b, lane 4). These results suggested that Jak2 kinase activity is required for tyrosine phosphorylation of EGFR in response to GH. G-protein-coupled receptors have been shown to mediate phosphorylation of EGFR through activation of Src tyrosine kinase[24], but GH did not stimulate the activity of Src kinase (data not shown). In addition, expression of Csk, which inactivates Src-family non-receptor tyrosine kinases by phosphorylating the regulatory carboxy-terminal tyrosine residue, reduced the Src kinase activity to only 20–30% of cells transfected with vector alone, whereas it had no apparent effect on GH-induced tyrosine phosphorylation of EGFR (data not shown). To clarify how GH induces phosphorylation of EGFR, we tested whether GH could induce the association of EGFR with GHR by co-immunoprecipitation experiments in COS cells transiently transfected with GHR and WT Jak2; we found that complex formation between GHR and EGFR was indeed induced in a GH-dependent manner (Fig. 4c, lane 2).

After GH binds to its receptor, the affinity of Jak2 for GHR appears to increase[2]. To determine whether EGFR recruited to GHR by GH stimulation could be directly phosphorylated by Jak2, we carried out immune-complex kinase assays in vitro. The GST-cytoplasmic region of WT EGFR was phosphorylated by Jak2 immunoisolated from CHO-GHR cell extracts treated with GH, and it was able to associate with Grb2 (Fig. 4d, lane 5). The amount of Jak2-induced phosphorylation of the GST–cytoplasmic region of the F4/Y1068-EGFR and its association with Grb2 were ~70–80%
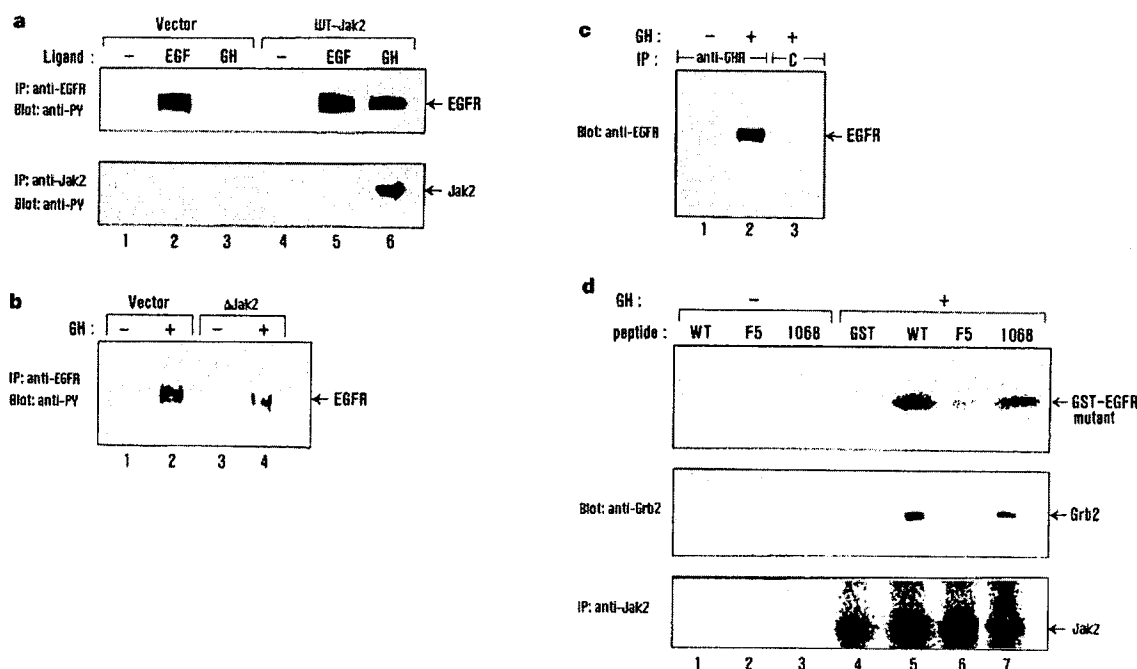
Figure 4 Tyrosine 1068 of EGFR was one of the major phosphorylation and Grb2-binding sites stimulated by GH via Jak2. **a-c**, WT EGFR plasmids were transfected into γ2A/GHR with or without WT Jak2 plasmids (**a**) or CHO-GHR with or without ΔJak2 plasmids (**b**). hGHR and WT Jak2 were cotransfected to COS cells (**c**). Quiescent cells were stimulated for 5 min with EGF (100 ng ml⁻¹) or hGH (500 ng ml⁻¹). Upon lysis, precipitates with anti-EGFR (**a**, top, **b**) or anti-Jak2 (**a**, bottom) or anti-GHR or control mouse IgG (C) were immunoblotted with anti-PY (**a, b**) or anti-EGFR (**c**). **d**, In vitro Jak2 kinase assay. Top, phosphorylation of GST-EGFR-mutant fusion proteins induced by Jak2 immunoisolated from untreated or hGH-treated CHO-GHR cells. Centre, association of phosphorylated GST-EGFR-mutant fusion proteins with Grb2. Bottom, autophosphorylation of Jak2. Quiescent cells were stimulated with or without hGH and in vitro Jak2 kinase assays and in vitro association assays were done as described in Methods.

of that obtained for GST–WT-EGFR (Fig. 4d, lanes 5, 7), whereas these were almost undetectable for F5-EGFR (Fig. 4d, lane 6), which is consistent with in vivo data (Fig. 3a). These results indicated that Tyr 1068 may be one of the principal phosphorylation and Grb2-binding sites induced by Jak2 in response to GH, although other tyrosine phosphorylation sites may also be able to mediate association with Grb2 (refs 18, 19).

The EGF receptor is known to be important in signalling pathways triggered by ultraviolet irradiation[25] and by G-protein-coupled receptors[26], but it was considered that the intrinsic kinase activity of EGFR was necessary for signal transduction[25,26]. More recently, it has been suggested that G-protein-coupled receptors may cause phosphorylation of EGFR by Src kinase, so independently of the intrinsic kinase activity of EGFR[24]. Together with these observations, our findings provide the new paradigm that non-tyrosine-kinase receptors transduce signals through an associated non-receptor tyrosine kinase by phosphorylating the growth-factor receptor and utilizing it as a docking protein independent of its receptor tyrosine kinase activity. □

Methods

Materials. Murine IL-6, monoclonal anti-phosphotyrosine antibody (4G10), polyclonal antibody against mouse Jak2 and polyclonal antibody against human EGFR were all from Upstate Biotechnology; monoclonal antibody against EGFR (E 3138), bovine GH and ovine prolactin (PRL) were from Sigma; monoclonal antibody against EGFR (mab c11) was from Life Technologies; polyclonal antibody against EGFR was from Santa Cruz Biotechnology; monoclonal anti-hGHR antibody (mab5) was from Agen Biomedical (Brisbane, Australia); human GH (hGH) was a gift from Eli Lilly; murine IFN-γ, lipofectin and lipofectamine were from Gibco BRL; murine LIF was from Amrad (Melbourne, Australia); and PD98059 was from Calbiochem. All other materials were from the sources given in ref. 15. Mice were treated as described[15].

EGFR mutants, adenovirus-mediated gene transfer and cell culture. EGFRs[19,28] were kindly provided by Y. Okabayashi and M. Kasuga. The recombinant adenoviruses Adex1CALacZ and Adex1CAEGFR were constructed by homologous recombination between the expression cosmid cassette and the parental virus genome[16] and adenovirus-mediated gene transfer was done as described[16]. CHO cells were transfected with plasmids and selected as reported[19] (~3.0 × 10⁴ GH receptors per cell). When the adenovirus Adex1CALacZ was applied at 3 × 10⁷ PFU cm⁻² per dish, LacZ was expressed in nearly 100% of CHO cells on post-infection days 1 and 2, and cells showed no significant changes, including in their morphology (data not shown). Thus, we applied recombinant adenoviruses at this dose and control cells were infected with Adex1CALacZ virus. INS-1 (ref. 17) and 3T3 F442A cells[11] were all grown as described.

MAP kinase assay, immunoprecipitation and immunoblotting. Immunoprecipitation, immunoblotting and MAP kinase assays have been described[15]. When immunoprecipitations were sequential, supernatants of liver homogenates from GH (5 μg per g body weight)-stimulated mice were immunoprecipitated with anti-EGFR antibody. Immune complexes were collected with protein G–Sepharose; supernatants were reprecipitated with anti-EGFR antibody. Following three rounds of anti-EGFR immunoprecipitation, supernatants were immunoprecipitated with 4G10 and the immunoprecipitates from each step were immunoblotted with 4G10.

Expression of wild-type and kinase-inactive Jak2, hGHR and Csk. WT EGFR cDNA (3 μg) in pRc/CMV was transfected into γ2A/GHR[5] or CHO-GHR cells with or without 3 μg WT Jak2 plasmids or dominant-negative Jak2 (ΔJak2; ref. 23), which lacks the C-terminal kinase domain under the SRα promoter, in 6-cm dishes by the lipofectamine method with modification[29] and the cells were stimulated as described[5]. The coding region of hGHR was isolated at NheI and MluI sites, and the insert was subcloned into pCI vector. COS cells were transfected with 3 μg hGHR, 2 μg WT Jak2 and 3 μg Csk at 60–80% confluence in 3 ml of DMEM medium in 6-cm dishes by the calcium phosphate precipitation method; cells were stimulated as described[3].

Generation of GST–EGFR fusion protein, in vitro Jak2 kinase assay, and

in vitro and *in vivo* **association assays.** The GST–cytoplasmic region of EGFR fusion proteins (amino acids 930–1,220, which do not include Lys 721, a key residue in the ATP-binding site) were generated as described[28]. The *in vitro* Jak2 assay was done as before[2], with minimal modification. Proteins immobilized on anti-Jak2 antibody were mixed with 95 μl of 0.1% Triton X-100 reaction buffer[2] containing 10 μM ATP, 0.25 mCi ml⁻¹ [γ-32P] ATP and 10 μg GST–EGFR-mutant fusion protein. After reaction, the anti-Jak2 immune complexes were resolved by SDS–PAGE and the autophosphorylation of Jak2 was visualized by autoradiography. To collect GST–EGFR-mutant fusion proteins, the supernatant was incubated with 30 μl glutathione–Sepharose beads; 32P-labelled proteins were then resolved by SDS–PAGE and phosphorylation of GST–EGFR-mutant fusion proteins was visualized by autoradiography. When the *in vitro* association of phosphorylated GST–EGFR-mutant fusion proteins with Grb2 was determined, instead of being visualized by autoradiography, the fusion proteins were incubated with Grb2, processed as described[19], and immunoblotted with anti-Grb2. To determine association of EGFR mutants with Grb2 *in vivo*, and the tyrosine-phosphorylation or MAP-kinase activity in cells expressing EGFR mutants, CHO-GHR cells were transfected with 6 μg of pRc/CMV alone or with WT or EGFR mutant cDNA in pRc/CMV in 6-cm dishes by using the lipofectamine method with modification[29]. Upon cell lysis, precipitated EGFR was immunoblotted with anti-PY (phosphotyrosine), and immunoprecipitates by anti-Grb2 were immunoblotted with anti-EGFR.

Luciferase assay. CHO-GHR cells were plated in 12-well dishes. One day after adenovirus-mediated gene transfer, cells were transfected by using the lipofectin procedure with 0.15 μg fusion gene construct pFOS(-359)Luc[30]. After incubation for 18 h with lipofectin in serum-free Ham's F-12 medium, transfected cells were pretreated for 60 min with or without 30 μM PD98059, then treated with hGH (500 ng ml⁻¹) for 5.5 h. Luciferase was assayed as described[20].

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Peptide bond formation by *in vitro* selected ribozymes

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An attractive solution to the problem of the origin of protein synthesis in an evolving 'RNA world' involves catalysis by nucleic acid without assistance from proteins[1,2]. Indeed, even the modern ribosome has been considered to be fundamentally an RNA machine[3], and the large ribosomal subunit can carry out peptidyl transfer in the absence of most of its protein subunits[4]. Successive cycles of *in vitro* selection and amplification[5–7] have been used to find RNAs that perform many biochemical reactions[8–16], including transfer of an RNA-linked amino acid to their own 5'-amino-modified terminus[15]. Here we demonstrate the *in vitro* selection of ribozymes (196 nucleotides) that perform the same peptidyl transferase reaction as the ribosome: that is, they can join amino acids by a peptide bond. Like ribosome substrates, one amino acid (N-blocked methionine) is esterified to the 3'(2')-O of adenosine, whereas the acceptor amino acid (phenylalanine) has a free amino group. Our best characterized ribozyme recognizes the amino-acid ester substrate by binding its adenosine moiety, and is therefore capable of utilizing Leu- and Phe- as well as Met-derived substrates. Such lack of specificity with respect to the amino acid is a feature necessary for a generalized protein-synthesizing enzyme.

We synthesized a pool of 1.3×10^{15} different RNA molecules containing two regions of randomized sequence (70 and 72 nucleotides) flanked by constant regions. The template for *in vitro* transcription was a synthetic DNA library constructed by using the polymerase chain reaction (PCR) (see Methods). Transcription was performed in the presence of guanosine-5'-monophosphorothioate (GMPS) to introduce a 5'-phosphorothioate. The 196-mer 5'-GMPS-RNA pool was then reacted with N-bromoacetyl-N'-phenylalanyl-cystamine to convert to 5'-Phe-SS-RNA. Thus, each RNA had a Phe at its 5' end that provided a free amino group capable of functioning as an aminoacyl acceptor (Fig. 1).

To select active RNA, the 5'-Phe-SS-RNA pool was incubated

Advances in Brief

Ultraviolet Radiation Induces Phosphorylation of the Epidermal Growth Factor Receptor[1]

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Abstract

Ultraviolet light in solar radiation is responsible for more than 600,000 malignancies each year in the United States alone, making it the most efficient environmental carcinogen known. Ultraviolet radiation-induced direct DNA damage is thought to be responsible for its initiating properties, while the promotional aspects of such radiation are poorly defined and only recently gaining attention. We show here for the first time that physiologically relevant doses of ultraviolet radiation induce phosphorylation of the epidermal growth factor receptor in A431 keratinocytes at tyrosine sites within 30 min. Such alteration of this major signal transduction system is probably an important step in the ultraviolet radiation-induced, epidermal cell-signalling cascade.

Introduction

UV radiation is capable of inducing both acute and chronic effects in human skin. These include inflammation and hyperpigmentation, hyperplasia of the epidermis and, most importantly, melanoma and nonmelanoma skin cancer. UV acts in the skin, at least for nonmelanoma skin cancer, as a complete carcinogen, and it has been shown to act, with appropriate chemical agents, as both a tumor initiator and a tumor promoter (1, 2). We have been interested in the promotion-related effects of UV, because UV irradiation of intact skin *in vivo* produces many effects analogous to those produced by the classic chemical tumor promoter TPA[3] (2). UV and TPA produce inflammation and proliferation in human and animal skin, both mediated in part by metabolites of arachidonic acid (3–5). Our previous investigations have shown that UV induces arachidonic acid release through phospholipase activation and eicosanoid production in murine and human fibroblasts and in human epidermal keratinocytes (6, 7). Others have reported similar observations (8, 9). A related early event in the cascade induced by TPA in cultured cells is the inhibition of EGF binding to its receptor (EGFR). This inhibition has been shown to be due to phosphorylation of EGFR at threonine sites by PKC, the receptor kinase for TPA (10). We have previously reported that UV induces a rapid inhibition of EGF binding in murine fibroblasts and human keratinocytes (11, 12). In other studies we examined the effect of UV on PKC activity and found that UV was capable of inducing

activity of the kinase (13). This suggested that UV inhibition of EGF binding might be due to PKC phosphorylation of EGFR.

Materials and Methods

Cell Culture System. Cryopreserved stocks of human epidermoid carcinoma cells (A431) were obtained from the American Type Culture Collection, Rockville, MD. The cells were grown to subconfluency in DMEM supplemented with 10% bovine fetal calf serum, penicillin (75 units/ml), and streptomycin (25 mg/ml). The cells were grown in a humidified atmosphere of 5% CO_2:95% air at 37°C. A431 cells in 10-cm dishes were treated with EGF, TPA, and DMSO and irradiated with UVB or sham irradiated in HBSS without phenol red.

Light Source and Irradiation. UVB irradiation was performed on monolayer cultures with dish lids removed, utilizing a light source composed of six Phillips TL20W/12 fluorescent Sun Tubes. The irradiance at a target distance of 25 cm was 12 W/m² measured with a Model IL700 International Light Research radiometer with a SEE 1240 probe. The spectral emission of the source was 290–360 nm with emission in the UVB range (290–320 nm) accounting for 60% of the irradiance.

Western Blotting. Cells were rinsed with ice-cold PBS and harvested in Laemmli buffer [143 mM Tris (pH 6.8):3% SDS:5% 2-mercaptoethanol:10% glycerol]. Equal amounts of protein from each sample were subjected to discontinuous polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) (14) in which the stacking gel was 4% acrylamide:bis(50:1) and the resolving gel was 8%. The separated proteins were then electrophoretically transferred overnight to a NC membrane. NC was washed and incubated with mouse anti-phosphotyrosine monoclonal IgG2bk antibody (UBI, Lake Placid, NY). Proteins containing phosphotyrosine were visualized with a goat anti-mouse IgG antibody linked to horseradish peroxidase (UBI, Lake Placid, NY).

Immunoprecipitation of EGFR. A431 cells were washed with phosphate-free DMEM and then starved for 4 h in phosphate-free DMEM containing 5% dialyzed calf serum. This conditioned medium was removed, supplemented with 0.07 mCi/ml of $^{32}P_i$ (Dupont, NEN Research Products, Boston, MA), and added back to the cells.

After 3 h the radioactive medium was removed, cells were washed twice with HBSS without Ca^{2+}, and HBSS with Ca^{2+} was added to the dishes. This was followed by irradiation, sham irradiation, or treatment with EGF (150 ng/ml) (Sigma, St. Louis, MO).

At selected times after incubation at 37°C, cells were washed twice with ice-cold PBS and harvested on ice in enriched PBS solution containing 10 ng/ml of leupeptin, 10 ng/ml of pepstatin A, 20 ng/ml of aprotinin, and 1 mM PMSF. Then cells were centrifuged, and the supernatant was discarded and resuspended in RIPA buffer. All subsequent steps were performed at 4°C according to a procedure modified from that of Stoscheck and Carpenter (15). The solution was centrifuged, and 15 μl of monoclonal mouse anti-human EGFR (UBI, Lake Placid, NY) were added per sample and incubated overnight. A 50% solution of Protein G (Sepharose Fast Flow 4B; Sigma Chemical Corp., St. Louis, MO) was added to each sample, and incubation continued for 2 h. Then the cell lysate was centrifuged, the supernatant was discarded, and the pellets were washed 4 times in RIPA buffer. The immunoprecipitated samples were boiled for 5 min in Laemmli buffer and then electrophoresed on a 7.5% SDS-PAGE gel.

The dried gel was exposed at −70°C to Kodak X-Omat AR film. The intensity of the bands obtained after autoradiography was quantified photodensitometrically with a computerized laser densitometer (Version 3.2, Image Quant, Series 300 densitometer; Molecular Dynamics, Sunnyvale, CA).
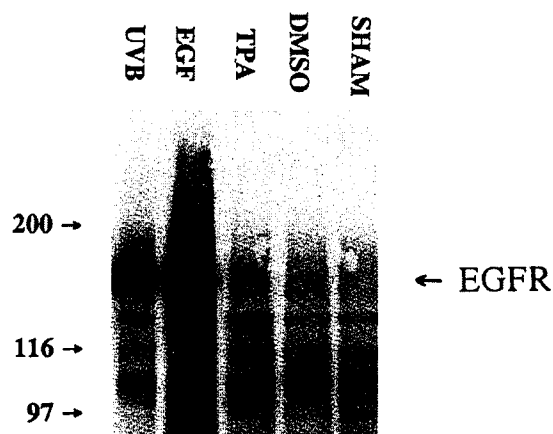
Fig. 1. The effect of UVB irradiation on protein tyrosine phosphorylation. A431 cells were treated with EGF (100 ng/ml), DMSO (2 μl/ml), and TPA (200 ng/ml) and irradiated with UVB (600 J/m²) or sham irradiated. Fifty min after treatment, cells were harvested, and proteins were extracted and separated by SDS-PAGE, transferred to NC membranes, blotted with anti-phosphotyrosine antibody, and visualized with secondary antibody by the horseradish peroxidase method.

Identification of Phosphoamino Acids. After irradiation, sham irradiation, or EGF treatment, cells were harvested and EGFR was immunoprecipitated (15). Phosphoamino acids were identified and quantitated using a procedure modified from that of Honegger et al. (16). Immunoprecipitated protein was transferred to hydrolysis tubes, dried with a Savant Speed Vac concentrator, then hydrolyzed, and placed in a reaction vial containing 6 N HCl at 110°C for 3 h under argon at approximately 1.5 torr.

After hydrolysis, samples were dried, dissolved in 30 μl of sample buffer (0.025% EDTA), and analyzed with an Applied Biosystems 420A derivatizer
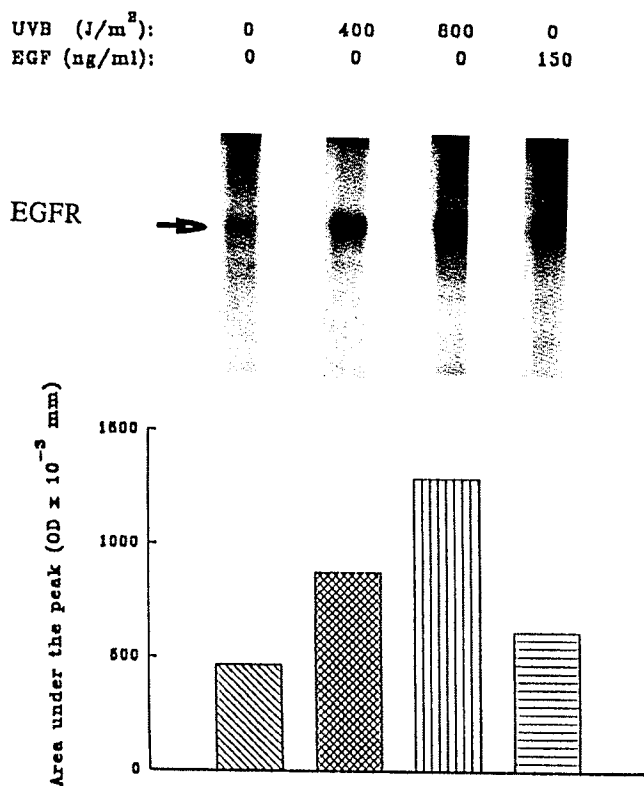

Fig. 2. UVB-induced phosphorylation of immunoprecipitated EGFR. A431 cells were preincubated with [³²P]orthophosphate and then irradiated with selected doses of UVB, sham irradiated, or treated with EGF (150 ng/ml). After 30 min, cells were harvested, and EGFR was immunoprecipitated. The immunoprecipitated proteins were separated on a 7.5% SDS-PAGE gel, and phosphorylated EGFR was identified by autoradiography. OD, absorbance.

coupled to an on-line 130A separation system (RP-HPLC) using precolumn derivatization followed by subsequent analysis of amino acids. Solutions of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) were used as internal standards.

Elution absorbance data were collected using a 760 interface and XTRA CHROM II data system (PE Nelson, Cupertino, CA). The derivatized phosphoamino acids were quantitated by comparing the sample absorbance peak areas.

Results and Discussion

We examined the effect of UVB (290–320 nm) on EGFR in A431 cells, which are derived from a human squamous cell carcinoma and which express high levels of EGFR. Cells were irradiated with UVB (600 J/m²), and extracted proteins were analyzed by immunoblotting with a monoclonal anti-phosphotyrosine antibody. As seen in Fig. 1, an immunoreactive protein with a molecular weight of approximately 170,000 was noted, corresponding to EGFR. A similar band was seen in EGF-treated but not sham-irradiated or TPA-treated cells. Preliminary studies revealed that this phosphorylation was maximal at 30 min postirradiation (data not shown).

We then examined specific characteristics of this phosphorylation. Cells were prelabeled with [³²P]orthophosphate and then irradiated with 400 and 800 J/m². Cell extracts were immunoprecipitated with monoclonal anti-EGFR antibody and separated by gel electrophoresis. A phosphorylated M_r 170,000 protein was identified in irradiated but not sham-treated cells (Fig. 2). A similar band was noted in EGF-treated cells. Densitometric analysis of the autoradiograms revealed a dose-related phosphorylation of EGFR, with 400 J/m² inducing twice and 800 J/m² almost 3 times the level of sham-irradiated cells.

A time course study shown in Fig. 3 revealed that the phosphorylation of the immunoprecipitated receptor in UVB-treated cells peaked somewhat later and persisted longer than in EGF-treated cells. To more clearly define the amino acid identity of the phosphorylated site induced by UVB, the immunoprecipitated receptor was hydrolyzed, and phosphorylated amino acids were identified by RP HPLC after derivatization (Fig. 4). UVB markedly increased the quantity of phosphorylated (p-) tyrosine residues in the EGFR hydrolysates, but had no effect on p-serine or p-threonine content. EGF

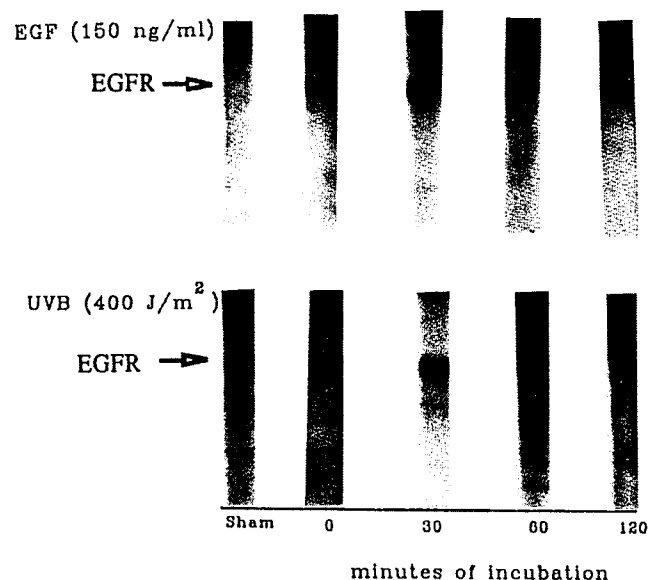minutes of incubation

Fig. 3. Time course of UVB-induced phosphorylation of immunoprecipitated EGFR. A431 cells were irradiated, sham treated, or treated with EGF as in Fig. 2. Beginning immediately after treatment, cells were harvested at the indicated times, EGFR was immunoprecipitated and separated by gel electrophoresis, and phosphorylation of EGFR was determined by autoradiography as in Fig. 2.
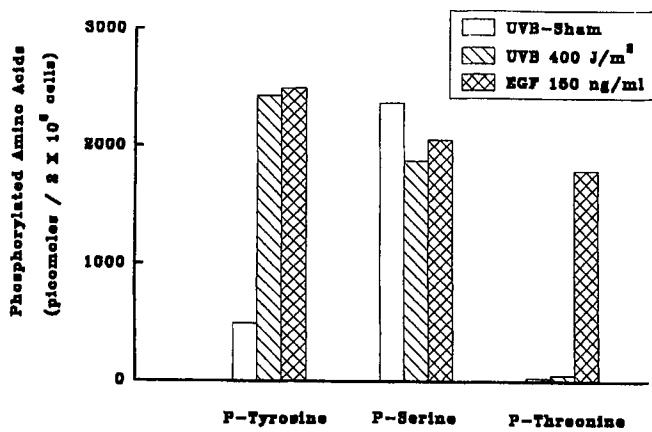
Fig. 4. The effect of irradiation on phosphoamino acid content of immunoprecipitated EGFR. After irradiation, sham treatment, or EGF treatment, cells were harvested and EGFR was immunoprecipitated. The EGFR was then hydrolyzed, and the phosphoserine, phosphothreonine, and phosphotyrosine contents of the samples were determined by RP-HPLC as outlined in "Materials and Methods."

treatment resulted in a similar increase in p-tyrosine but unlike UVB also increased p-threonine, consistent with data reported previously in this cell type (17).

Others have examined the effect of UVB on EGFR. Brooks et al. (18) reported that UVB-induced inhibition of ^{125}I-EGF binding in murine fibroblasts and keratinocytes was not blocked by depletion of PKC with TPA treatment and was not associated with phosphorylation of the M_r 80,000 protein substrate of PKC. They concluded that the UVB-induced inhibition, therefore, was by a PKC-independent mechanism. Our finding of tyrosine rather than serine or threonine phosphorylation in UVB-treated cells supports their view. We have also reported that neither TPA-induced depletion of PKC nor treatment with the PKC inhibitors H7 and staurosporine affected UVB-induced inhibition of ^{125}I-EGF binding in C3H10T½ cells (17).

When EGF binds to EGFR, receptor dimerization and autophosphorylation of tyrosine sites occur, which condition leads to phosphorylation of substrates such as phospholipase C. This begins a cascade of signal transduction which results in proliferation of most cells. Interestingly enough, this cascade includes PKC activation which probably acts to exert a later, negative control on EGFR activity through phosphorylation at threonine sites (19). Activation of EGFR would be expected to lead to hyperplasia in vivo, as occurs after a UVB-induced "sunburn," and, in fact, increased EGFR expression has been reported in hyperplastic epidermis of psoriasis (20).

The mechanism by which UVB stimulated phosphorylation of EGFR is at this point undefined. TGF-α, which also binds to EGFR, has been shown to be secreted by UVB-treated cultured cells. In their studies, Brooks et al. (18) investigated the effect of UVB-induced, soluble, excreted factors on EGF binding and found no effect, suggesting that UVB-induced inhibition of binding was not through TGF-α production. UVB-induced phosphorylation is rapid, occurring within 30 min of irradiation. This suggests a direct photon-membrane interaction. It is possible that UVB causes dimerization and autophosphorylation of EGFR through a photochemical reaction. Activation of other tyrosine kinases, however, cannot be ruled out.

The doses of radiation capable of inducing receptor phosphorylation (400 J/m² measured as described) can be received at the skin

surface in New York City in July within 5 to 10 min of outdoor activity. Several consequences of UVB-induced EGFR phosphorylation may be physiologically important to the epidermal responses observed after irradiation. For example, activation of tyrosine kinase may be a critical early event in the induction of phospholipase activity, which is involved in the UV-induced inflammatory response (4). Postinflammatory hyperplasia and tanning may also be linked to kinase activation.

In addition, the ability of UVB to alter such an important membrane signal transducer as EGFR suggests that membrane second messengers are intimately involved in the cascade of events that leads to late effects of solar damage in human skin like carcinogenesis and photoaging. Finally, the effect of UVB on EGFR may play a role in the mechanism by which UVB phototherapy benefits psoriasis, a disease with abnormal, excessive EGFR expression (20).

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Activation of the Epidermal Growth Factor Receptor by Skin Tumor Promoters and in Skin Tumors from SENCAR Mice[1]

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Abstract

The present study was designed to further investigate the
role of the epidermal growth factor receptor (EGFr) in
mouse skin tumor promotion by evaluating the status of
the EGFr in tumor promoter-treated mouse epidermis
and in mouse skin tumors. Female SENCAR mice
received three topical treatments of either the phorbol
ester 12-O-tetradecanoylphorbol-13-acetate (TPA) or the
nonphorbol esters okadaic acid and chrysarobin.
Membrane proteins from SENCAR mouse epidermis
were isolated 6 h after the last treatment, and the
phosphotyrosine content of the EGFr and several
potential substrates were examined by Western blot
analysis. The results indicated that multiple applications
of all three tumor promoters led to an increase in the
phosphotyrosine content of the EGFr and also of several
lower molecular weight proteins (M_r ~80,000–85,000).
Phosphorylation of PLCγ1 on tyrosine residues could not
be detected in tumor promoter-treated mouse epidermis
when the phosphotyrosine content of the EGFr was
elevated or in cultured keratinocytes exposed to
exogenous EGF. When two tyrosine kinase inhibitors
(tyrphostins RG50864 and RG13022) were incorporated
into the treatment regimens, the TPA-induced epidermal
hyperplasia and cell proliferation were effectively
blocked, and the TPA-stimulated EGFr tyrosine
phosphorylation was significantly reduced. Examination
of the phosphotyrosine content of epidermal membrane
proteins isolated from skin papillomas revealed that the
EGFr also had elevated phosphotyrosine levels. These
results demonstrate that multiple topical treatments with
both phorbol ester and nonphorbol ester tumor
promoters lead to activation of the EGFr tyrosine kinase
in mouse epidermis. In addition, these data suggest that
signaling through the EGFr pathway plays an important
role in the tumor promotion stage of multistage
carcinogenesis in mouse skin.

Introduction

Topical treatment of mouse skin with tumor promoters such
as TPA[4] induces a hyperplasia characterized by increased
mitotic activity in the basal cell layer and an increased
number of suprabasal cell layers (1, 2). Specific cellular and
biochemical responses have been associated with the pro-
cess of skin tumor promotion primarily through studies of
the phorbol ester, TPA (3–6). TPA, by its interaction with
PKC, is believed to produce a number of cellular, biochem-
ical, and molecular changes leading to the process of skin
tumor promotion (7–9). In addition to phorbol esters, a wide
variety of chemically diverse compounds are known to
possess tumor-promoting properties in mouse skin, includ-
ing teleocidins, aplysiatoxins, anthrones, thapsigargin, OA,
and benzoyl peroxide (reviewed in Ref. 10). All known skin
tumor promoters induce a sustained and potentiated epi-
dermal hyperplasia following multiple topical treatments (3,
4, 6). In initiation-promotion protocols, prolonged and re-
peated application of a tumor promoter at appropriate in-
tervals to mice that have received previously a single dose
of carcinogen (initiation) results in a high yield of skin
papillomas, some of which will progress to squamous cell
carcinomas (2–4, 11). It is generally accepted that all skin
tumor-promoting agents ultimately lead to the selective
clonal expansion of initiated cells, although the exact bio-
chemical and molecular mechanisms may differ among
promoting stimuli (6, 7, 11). Although genetic mechanisms
have been postulated to play a role in the tumor promotion
stage (reviewed in Ref. 10), the eventual reversibility of
promoter-induced effects (12, 13) argues that this stage is
accomplished primarily through epigenetic mechanisms as
originally postulated by Boutwell (14) and Raick et al. (1).

RTKs are involved in growth control, and alterations in
several of these ligand/receptor systems may result in un-
controlled growth and oncogenic transformation (15, 16).
One of the best studied RTKs is the EGFr. The EGFr has a
molecular mass of 170 kilodaltons and is composed of a
single polypeptide chain of 1186 amino acid residues with
~40 kilodaltons of N-linked oligosaccharides (17–19). A
single hydrophobic transmembrane sequence separates an
extracellular ligand binding domain from a cytosolic do-
main (17–19). The activation of intrinsic tyrosine kinase
activity of the EGFr following the binding of ligands, such as
EGF or TGF-α, appears to be required for the diverse bio-
logical responses of the receptor (19, 20).

TGF-α is a potent mitogen for cultured keratinocytes (21,
22). Several types of skin tumor promoters have been shown
to induce TGF-α mRNA expression and protein levels in

cultured keratinocytes as well as in mouse epidermis *in vivo* (reviewed in Ref. 10). Recent studies from our laboratory have shown that four distinct classes of skin tumor promoters (23, 24) and full thickness skin wounding induce epidermal TGF-α mRNA and protein synthesis (24).[5] Transfection of a human TGF-α gene into cultured primary mouse keratinocytes and then grafting these cells to nude mice leads to growth of tumors larger than those obtained with untransfected papilloma cells alone (25). In other studies, overexpression of TGF-α in epidermis of transgenic mice, where expression was targeted to epidermal basal cells, resulted in a phenotype displaying marked epidermal hyperplasia (26, 27). Collectively, these observations support the hypothesis that TGF-α (and hence, signaling through the EGFr) plays an important role during the tumor promotion stage of skin carcinogenesis. Furthermore, we recently reported that both TGF-α and EGFr mRNA and protein are constitutively overexpressed in skin papillomas induced by initiation-promotion regimens in SENCAR mice (28). Thus, constitutive activation of the EGFr tyrosine kinase may also be important in the development of autonomous tumor growth in this model system.

In the present study, we evaluated the status of the EGFr in tumor promoter-treated mouse epidermis and in mouse skin tumors. We report that topical treatment with several distinct classes of skin tumor promoters leads to activation of the EGFr as measured by a net increase in phosphotyrosine content. Furthermore, we show that a tyrosine kinase inhibitor blocks tumor promoter-induced activation of the EGFr and tumor promoter-induced cell proliferation *in vivo*. These data demonstrate for the first time that activation of the EGFr tyrosine kinase occurs *in vivo* in mouse epidermis exposed to tumor promoters and that signaling through this RTK pathway plays an important role in the tumor promotion stage of multistage carcinogenesis in mouse skin.

Results

Elevated Phosphotyrosine Level of the EGFr following Multiple Treatment with TPA.
The initial experiments were designed to examine the effect of topical application of TPA on the phosphotyrosine level of the EGFr in mouse epidermal membrane preparations. Mice were treated with TPA (3.4 nmol) three times at the same interval used in skin tumor promotion experiments and sacrificed 6 h after the last treatment. In previous experiments (data not shown), it was determined that 6 h was an optimum time for measuring the phosphotyrosine content of the EGFr following TPA treatment *in vivo*. Control mice were treated with the acetone vehicle using a similar protocol. Plasma membrane was isolated (see "Materials and Methods") and subjected to SDS-PAGE and Western blot analysis using an antibody against phosphotyrosine (PY20; Fig. 1, Lanes 3 and 4). An increase in phosphotyrosine level was detected in a protein band with a molecular weight of M_r 175,000 in TPA-treated samples relative to the control samples. Other increases in phosphotyrosine levels occurred in protein bands with molecular weights of M_r ~80,000–85,000. Western blot analysis of the same tissue samples in which an antibody against the EGFr was used revealed that the tyrosine-phosphorylated band at M_r 175,000 migrated in the same position as the EGFr (Fig. 1, Lanes 1 and 2). In addition, these data
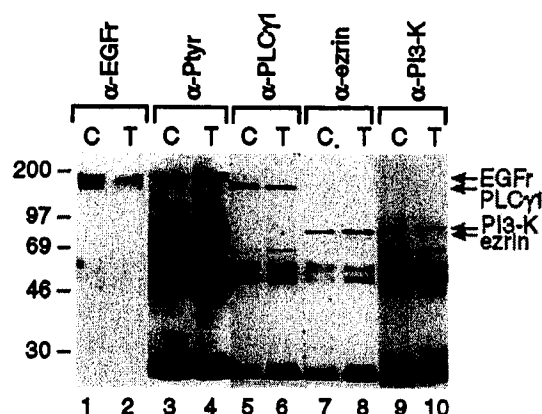
Fig. 1. Western blot analysis of the phosphotyrosine level of the EGFr and several putative EGFr substrates in epidermal membrane preparations following multiple treatments with TPA. Mice were treated topically with three applications of acetone (C) or 3.4 nmol TPA (T) and killed 6 h after the last treatment. Plasma membranes were isolated from mouse epidermis, and equal amounts of membrane proteins (10–25 μg/lane) were subjected to 7% SDS-PAGE and Western blot analysis as described in "Materials and Methods." Lanes 1 and 2 were probed with polyclonal anti-EGFr antibody (RK2); Lanes 3 and 4 were probed with monoclonal anti-phosphotyrosine antibody (PY20); Lanes 5 and 6 were probed with monoclonal anti-PLCγ1 antibody; Lanes 7 and 8 were probed with monoclonal anti-ezrin antibody; Lanes 9 and 10 were probed with monoclonal anti-PI3-K antibody.

show that TPA treatment of mouse epidermis did not lead to significant increases in EGFr protein levels 6 h after the last treatment.

It has been shown in many studies using cells in culture that activation of the EGFr tyrosine kinase leads to tyrosine phosphorylation of several intracellular substrates (29). Several of these substrates are known to have molecular weights in the range of M_r 80,000–85,000, including ezrin (30) and PI3-K (31). Therefore, we decided to determine whether these proteins were present in our membrane preparations. In addition, we also analyzed the same samples for the presence of PLCγ1. Western blotting of the membrane preparations showed that ezrin (M_r ~80,000) was present and expressed at approximately similar levels in both control and TPA-treated samples. Ezrin migrated in a position similar to one of the protein bands exhibiting increased tyrosine phosphorylation (Fig. 1, Lanes 7 and 8). Western blotting of membrane proteins also showed that PI3-K (M_r ~85,000) was expressed at similar levels in both control and TPA-treated samples and also migrated in a position similar to one of the bands exhibiting increased tyrosine phosphorylation (Fig. 1, Lanes 9 and 10). Finally, we also detected PLCγ1 (M_r ~150,000) in our epidermal membrane preparations (Fig. 1, Lanes 5 and 6). PLCγ1 protein levels were similar in both control and TPA-treated samples. Interestingly, no bands were detected at a position with a molecular weight similar to PLCγ1 in the samples (control- or TPA-treated) that were immunoblotted with PY20 (Fig. 1, Lanes 3 and 4).

To further verify that the protein band at molecular weight M_r 175,000 showing increased phosphotyrosine levels in TPA-treated tissues was the EGFr, membrane proteins (500 μg) from both control and treated tissues were immunoprecipitated with an antisera (1382) against the extracellular domain of rat EGFr bound to protein A-agarose. After SDS-PAGE, the immunoprecipitates were probed

A

α-EGFr α-Ptyr

C T | C T

200 –

97 –

69 –

46 –

1 2 3 4

← EGFr

B

α-PLCγ1 α-Ptyr

C T | C T

200 –

97 –

69 –

46 –

1 2 3 4

← PLCγ1

Fig. 2. Western blot analysis of the phosphotyrosine level of the EGFr (A) and PLCγ1 (B) following immunoprecipitation. Mice were treated topically with three applications of acetone (C) or 3.4 nmol TPA (T) and killed 6 h after the last treatment. Equal amounts (500 µg) of microsomal membrane protein were solubilized in lysis buffer and immunoprecipitated with a polyclonal antibody against EGFr (1382) or PLCγ1 as described in "Materials and Methods." The immunoprecipitates were subjected to 7% SDS-PAGE and Western blot analysis. A, Lanes 1 and 2, were probed with anti-EGFr antibody (RK2), and Lanes 3 and 4 were probed with anti-phosphotyrosine antibody (PY20). B, Lanes 1 and 2, were probed with anti-PLCγ1 antibody, and Lanes 3 and 4 were probed with anti-phosphotyrosine antibody (PY20).

with a polyclonal anti-EGFr antibody (RK2) and a monoclonal anti-phosphotyrosine antibody. As shown in Fig. 2A, the EGFr was tyrosine-phosphorylated, and the phosphotyrosine level of the EGFr in the TPA-treated sample was approximately 3-fold higher than that of the control sample. This increase accounted for ~90% of the increase in phosphotyrosine level in the M_r 175,000 protein detected by Western blotting of the total membrane proteins (Fig. 1, Lanes 3 and 4). Also, as shown in Fig. 2A, Lanes 1 and 2, the EGFr protein level was unchanged, which was consistent with the results of the experiments in which plasma membrane preparations were analyzed (Fig. 1, Lanes 1 and 2). These results confirm that multiple TPA treatments induce an elevation in the phosphotyrosine level of the EGFr.
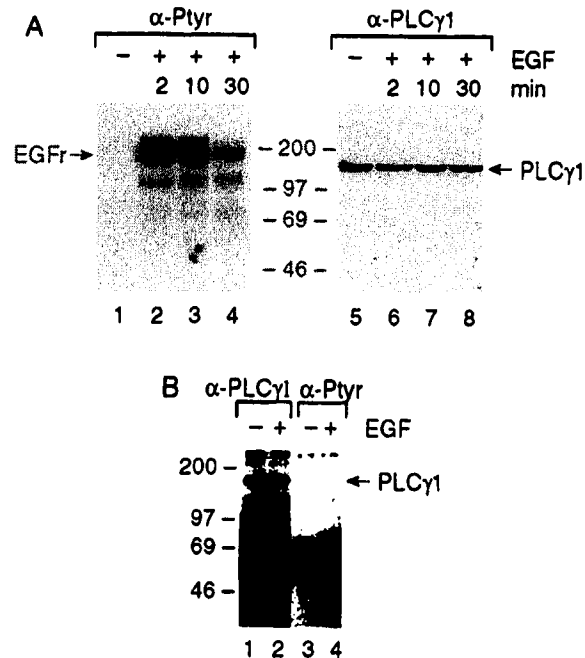
A

α-Ptyr   α-PLCγ1

– + + +   – + + + EGF

2 10 30   2 10 30 min

EGFr→

– 200 –

– 97 –

– 69 –

– 46 –

← PLCγ1

1 2 3 4   5 6 7 8

B

α-PLCγ1 α-Ptyr

– + – + EGF

200 –

97 –

69 –

46 –

← PLCγ1

1 2 3 4

Fig. 3. Western blot analysis of PLCγ1 from a mouse keratinocyte lysate. Mouse epidermal basal cells were isolated and cultured in low Ca^{2+} MEM-2 medium with 1% FBS and supplements. Cells were starved for 24 h at day 3, stimulated with EGF for 2–30 min, and lysed in lysis buffer. Cell lysates (35 µg of protein) were subjected to SDS-PAGE and Western blot analysis (A) or subjected to immunoprecipitation using an anti-PLCγ1 antibody prior to SDS-PAGE and Western blot analysis (B). A, Lanes 1–4, were probed with anti-phosphotyrosine antibody (PY20), and Lanes 5–8 were reprobed with anti-PLCγ1 antibody after stripping the blot corresponding to Lanes 1–4. B, control sample (–) or EGF-stimulated (10 min) sample (+) was probed with anti-PLCγ1 antibody (Lanes 1 and 2) or PY20 (Lanes 3 and 4).

Further Analysis of PLCγ1 Phosphorylation State in Mouse Keratinocytes. As shown in Fig. 1, Lanes 5 and 6, PLCγ1 protein levels were similar in both control and TPA-treated samples. However, tyrosine phosphorylation of a protein with the appropriate molecular weight for PLCγ1 was undetectable by Western blotting with an anti-phosphotyrosine antibody in the initial membrane preparation (Fig. 1, Lanes 3 and 4). Epidermal membrane proteins from control and TPA-treated mice were further subjected to immunoprecipitation with a polyclonal anti-PLCγ1 antibody, followed by Western blotting with either an anti-phosphotyrosine or anti-PLCγ1 antibody. As shown in Fig. 2B, Lanes 3 and 4, no detectable phosphotyrosine associated with PLCγ1 was evident in either the control or TPA-treated samples at the 6-h time point.

To further explore the relationship between signaling through the EGFr and PLCγ1, we examined the tyrosine phosphorylation of PLCγ1 following EGF stimulation in cultured mouse keratinocytes. For these experiments, we used primary adult keratinocytes from SENCAR mice. Cells were stimulated with 40 ng/ml of EGF for 2–30 min after having been starved for 24 h. After various treatment periods, cells were lysed in lysis buffer. Thirty-five µg of total protein were subjected to SDS-PAGE, followed by Western blotting with an anti-phosphotyrosine antibody (Fig. 3A, Lanes 1–4). A dramatic increase in the phosphotyrosine content of the EGFr occurred as early as 2 min after EGF

stimulation, followed by a significant decrease in phosphorylation of the EGFr after 30 min. In contrast, the anti-phosphotyrosine antibody failed to detect a phosphotyrosine-containing protein in the correct molecular weight range for PLCγ1. However, PLCγ1 was clearly present and detectable in these preparations, as indicated in Fig. 3A, *Lanes 5–8.* PLCγ1 was also immunoprecipitated with a polyclonal anti-PLCγ1 antibody from cell lysates obtained 10 min after EGF stimulation. Western blot analysis of these samples with the anti-phosphotyrosine antibody revealed that no phosphotyrosine was detectably associated with PLCγ1 at this time point (Fig. 3B). Similar results were obtained from Western blot analyses of mouse epidermal membrane proteins stimulated *in vitro* with EGF (data not shown).

Effects of Other Tumor Promoters on EGFr Phosphorylation *in Vivo* and EGFr Status in Skin Tumors. To investigate whether an elevation of phosphotyrosine level of the EGFr is a common phenomenon following tumor promoter treatment, two non-phorbol ester skin tumor promoters were examined for their effects. Mice were treated three times with either OA (2.5 nmol) or Chr (220 nmol) and sacrificed 6 h after the last treatment. Plasma membrane proteins were isolated and subjected to SDS-PAGE and Western blot analysis using the anti-EGFr and anti-phosphotyrosine antibodies. The results of these experiments are shown in Fig. 4, *A* and *B*. The EGFr protein level was unchanged after either OA or Chr treatment (Fig. 4, *A* and *B*, *Lanes 1* and *2*, respectively). However, there was an apparent increase in the phosphotyrosine level of a M_r 175,000 protein and in proteins with molecular weights of M_r ~80,000–85,000. Western blot experiments with immunoprecipitated EGFr from the membrane proteins confirmed that, as is the case with TPA, the phosphotyrosine level of the EGFr was increased following treatment with OA and Chr (data not shown).

Recent studies from our laboratory have shown that TGF-α mRNA and protein are constitutively overexpressed in both papillomas and squamous cell carcinomas produced by initiation-promotion regimens in SENCAR mice (28). These data suggest that the EGFr may be constitutively stimulated in skin tumors. Therefore, we examined the tyrosine phosphorylation state of the EGFr in skin papillomas. Mice were initiated with DMBA and promoted with TPA and then sacrificed after 18 weeks of promotion. Papillomas from 3–4 mice were pooled, and membrane proteins were isolated. Fifty µg of membrane proteins were subjected to SDS-PAGE and Western blot analysis (Fig. 4C). The phosphotyrosine level of the EGFr was dramatically increased (>6-fold) in papillomas. Western blotting of the immuno-precipitated EGFr from papilloma membrane proteins yielded similar results. Fig. 5 summarizes the relative phosphotyrosine levels of the EGFr in tumor promoter-treated skin and papillomas compared to control skin based on densitometric quantitation of data from direct analysis of plasma membrane preparations.

Effect of Tyrphostins on TPA-induced Epidermal Hyperplasia. In light of our results showing activation of the EGFr in tumor promoter-treated skin, we evaluated the ability of two tyrosine kinase inhibitors, with some specificity for the EGFr tyrosine kinase (tyrphostins RG13022 and RG50864), to block TPA-induced epidermal hyperplasia and cell proliferation in SENCAR mice. Mice were treated with two applications of TPA (3.4 nmol) 72 h apart. Forty µg of tyrphostin (RG13022 or RG50864) were topically applied
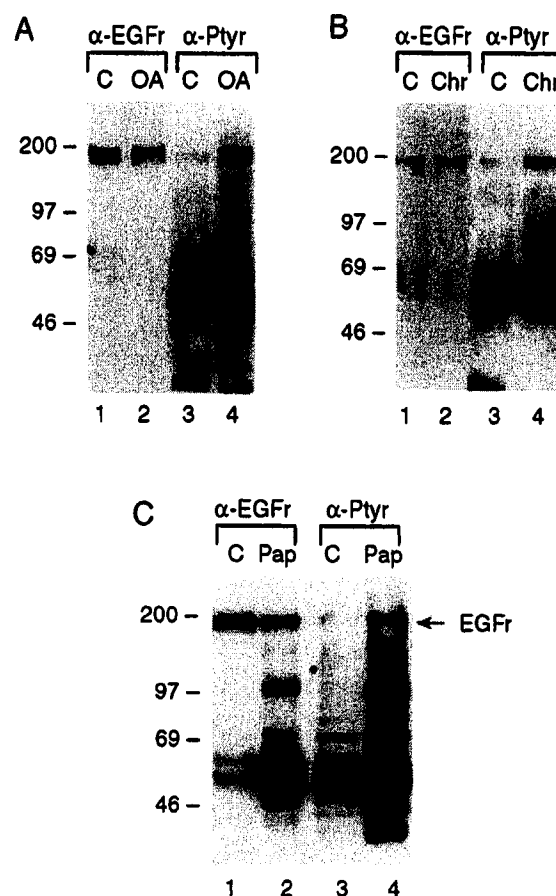
Fig. 4. Western blot analysis of the phosphotyrosine level of the EGFr in OA-treated epidermis (*A*), Chr-treated epidermis (*B*), and in mouse skin tumors (*C*). Mice were treated topically with three applications of acetone (*C*), 2.5 nmol OA, or 220 nmol Chr and killed 6 h after the last treatment. Papillomas from DMBA-initiated, TPA-promoted mice were harvested after 18 weeks of promotion and at least 2 weeks had passed since the last tumor promoter treatment. Equal amounts of epidermal plasma membrane protein (25–50 µg/lane) were subjected to SDS-PAGE and Western blot analysis. In all panels, *Lanes 1* and *2* were probed with anti-EGFr antibody (RK2), while *Lanes 3* and *4* were probed with anti-phosphotyrosine antibody (PY20).

10 min before and 12, 24, and 36 h after each TPA treatment. Mice were sacrificed 48 h after the last TPA treatment, and the skin was excised for histological evaluation of epidermal thickness and epidermal labeling index (the latter assessed by [³H]thymidine incorporation). As shown in Table 1, TPA produced significant epidermal hyperplasia compared to control mice treated with acetone as measured by epidermal thickness (54.1 ± 2.7 µm *versus* 14.0 ± 1.9 µm, respectively) and labeling index (36.7 ± 3.7 *versus* 4.2 ± 0.6 labeled nuclei/100 basal cells, respectively). Both RG13022 and RG50864 significantly reduced the TPA-induced epidermal hyperplasia by 55 and 43%, respectively, and labeling index by 92 and 75%, respectively. Neither of these parameters was significantly affected by tyrphostin treatment alone.

Experiments were also performed to determine whether the EGFr tyrosine kinase was blocked by treatment with the tyrphostin. For these experiments, tyrosine phosphorylation
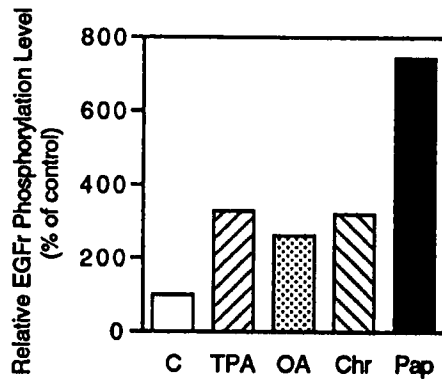
Fig. 5. Phosphotyrosine level of the EGFr in mouse epidermis following multiple treatments with different tumor promoters and in mouse skin tumors. Membrane proteins were isolated and analyzed as described in Figs. 1 and 4. The phosphotyrosine level of the EGFr was normalized to relative EGFr levels in various samples.

Table 1 Effect of tyrphostins RG13022 and RG50864 on TPA-induced hyperplasia and cell proliferation in mouse epidermis

Promoter	Tyrphostin	Epidermal thickness (µm)	Labeling index (labeled nuclei/ 100 basal cells)
–[a]	–	14.0 ± 1.9	4.2 ± 0.6
	RG13022	15.0 ± 1.3	3.5 ± 0.7
	RG50864	11.7 ± 1.4	3.8 ± 0.6
TPA	–	54.1 ± 2.7	36.7 ± 3.7
	RG13022	32.3 ± 4.5[b,c]	6.7 ± 0.3[b,c]
	RG50864	37.3 ± 4.9[b,d]	12.4 ± 4.9[b,c]

[a] –, absence of.
[b] Significantly different from the group treated with TPA alone ($P < 0.01$, ANOVA-Tukey test).
[c] Significantly different from the group treated with TPA alone ($P < 0.01$, two-sample t test when the variances are not assumed equal).
[d] Significantly different from the group treated with TPA alone ($P < 0.05$, two-sample t test when the variances are not assumed equal).

of the EGFr in TPA-treated skin was examined by Western blot analysis as described in Fig. 1. Mice were treated with TPA alone and TPA and tyrphostin RG13022 using a treatment protocol identical to that used for the histological assessment of epidermal proliferation. Control mice received the acetone vehicle in place of TPA. Membrane proteins were isolated and subjected to SDS-PAGE and Western blot analysis with the anti-EGFr and anti-phosphotyrosine antibodies. The results, after quantitation, are summarized in Fig. 6. As shown in Fig. 6, the EGFr phosphotyrosine level was increased 4-fold by TPA treatment in this experiment. When RG13022 was applied in conjunction with TPA, there was a dramatic reduction (~90%) in the TPA-stimulated EGFr tyrosine phosphorylation.

Discussion

The present study has provided direct evidence that the EGFr tyrosine kinase becomes activated in tumor promoter-treated skin *in vivo*. The major findings are: (a) multiple applications of TPA led to increased phosphotyrosine content of the EGFr and several lower molecular weight proteins (M_r ~80,000–85,000) in epidermal membrane preparations; (b) phosphorylation of PLCγ1 on tyrosine residues
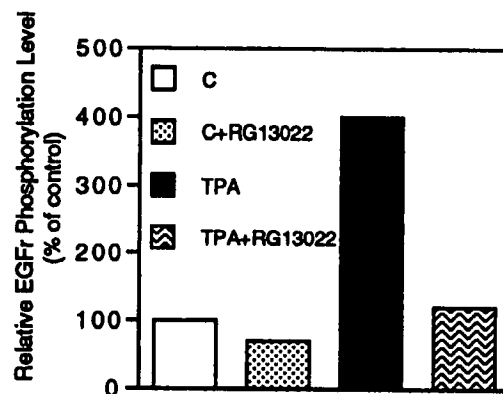

Fig. 6. Effect of tyrphostin RG13022 on elevated EGFr phosphotyrosine induced by TPA treatment. Mice were treated with two applications of 3.4 nmol TPA given 72 h apart. Forty µg of tyrphostin (RG13022) were topically applied 10 min before and 12, 24, and 36 h after each TPA treatment. Control mice received acetone in place of the tyrphostin or TPA. Mice were killed 48 h after the last TPA treatment. Plasma membrane proteins were isolated and analyzed as described in Fig. 1. Phosphotyrosine level of the EGFr was normalized to relative EGFr levels in appropriate samples.

could not be detected in tumor promoter-treated mouse epidermis when the phosphotyrosine content of the EGFr was elevated; (c) phosphorylation of PLCγ1 on tyrosine residues also could not be detected in cultured keratinocytes exposed to EGF; (d) multiple topical treatments with the nonphorbol ester promoters, Chr and OA, also led to an elevation in phosphotyrosine content of the EGFr in epidermal membrane preparations; (e) RG13022 and RG50864, tyrosine kinase inhibitors of the tyrphostin type, effectively blocked TPA-induced epidermal hyperplasia and cell proliferation and reduced TPA-stimulated EGFr tyrosine phosphorylation; and (f) the EGFr isolated from skin papillomas also had elevated phosphotyrosine. Taken together, the current data strongly support a role for signaling through the EGFr in tumor promotion by phorbol esters and possibly other classes of tumor promoters. In addition, the current data also extend our previous observations (28) that TGF-α mRNA and protein were elevated in skin tumors by now showing that the EGFr tyrosine kinase is constitutively activated in these same skin tumors.

A number of recent studies also indirectly support an important role for the EGFr during tumor promotion and multistage carcinogenesis in mouse skin. Imamoto *et al*. (23) reported that topical application of TPA and Chr led to the loss of epidermal PKC, elevated [^{125}I]EGF binding to EGFr, and induction of epidermal TGF-α mRNA and protein. Kiguchi *et al*. (24) further reported that topical application of OA and thapsigargin also led to elevated TGF-α mRNA and protein in mouse epidermis. As noted above, Rho *et al*. (28) recently reported that the expression of TGF-α was elevated in primary papillomas and squamous cell carcinomas induced by an initiation-promotion regimen. Jhappan *et al*. (32) reported that DMBA-initiated TGF-α transgenic mice, but not DMBA-treated control mice, developed skin tumors in the absence of further treatment. In this latter study, TGF-α transcripts were enhanced 10- to 20-fold in those tumors expressing the wild-type H-ras gene. The last two studies suggest that strong constitutive EGFr stimulation through TGF-α overexpression is

important for later stages of skin tumorigenesis (*i.e.,* tumor promotion). In addition to these studies, recent evidence from transfection experiments, where TGF-α was overexpressed in cultured keratinocytes (25), and the development of transgenic mice overexpressing TGF-α in basal keratinocytes (26, 27) also provide indirect support for a role of the EGFr signal transduction pathway, not only in later stages of chemically mediated skin carcinogenesis (*i.e.,* tumor promotion) but also in early stages (*i.e.,* initiation) of this process as well. In this regard, transgenic mice overexpressing TGF-α in basal keratinocytes developed tumors at sites of wounding or following exposure to TPA (33, 34). Most of these tumors did not possess mutations in the c-Ha-*ras* gene. Thus, since the EGFr is upstream of *ras*, these data suggest that constitutive stimulation of the EGFr tyrosine kinase can functionally substitute for c-Ha-*ras* activation (33, 34).

The role of signaling through the EGFr in mouse skin tumor promotion was further elucidated in the present study by demonstrating the inhibitory effects of tyrosine kinase inhibitors on promoter-induced activation of the EGFr and cell proliferation *in vivo.* Tyrphostins RG13022 and RG50864 are potent tyrosine kinase inhibitors that have been reported to have some selectivity for the EGFr (35–37). Our data demonstrated that topical application of tyrphostins RG13022 and RG50864 to mouse skin prior and after each TPA treatment effectively blocked TPA-induced epidermal hyperplasia as measured by epidermal thickness and epidermal labeling index (Table 1). TPA-induced elevation of the EGFr phosphotyrosine content was also effectively blocked by RG13022 (Fig. 6). These results are consistent with a study by Reddy *et al.* (36) showing that RG13022 inhibited EGFr autophosphorylation and EGF-stimulated breast cancer cell proliferation. However, as pointed out by these authors, RG13022 also inhibited growth that was stimulated by several unrelated growth factors such as insulin, insulin-like growth factor-I, and insulin-like growth factor-II. Therefore, we cannot rule out the possibility that the tyrosine kinase activities of other growth factor receptors and/or nonreceptor type tyrosine kinases critical for tumor promoter-induced cell proliferation were also inhibited by this tyrphostin.

The exact mechanism(s) for tumor promoter-induced EGFr activation as measured by increased phosphotyrosine content will require further investigation. Several mechanisms remain possible at the present time: (*a*) the increase in positive signal(s) for the EGFr leads to activation of the EGFr in tumor promoter-treated epidermis. As mentioned above, overexpression of TGF-α, a major ligand for EGFr, is induced by various tumor promoters in mouse epidermis (23, 24) and in skin tumors (28). The overexpression of TGF-α may provide sufficient positive signals for autocrine/paracrine stimulation of the EGFr; (*b*) the removal of negative feedback for the EGFr may lead or contribute to the activation of the EGFr. It has been well-documented that PKC phosphorylates EGFr at Thr-654 (38) and that this serves as a negative regulator of the EGFr tyrosine kinase (38, 39). Recent studies from our laboratory have shown that PKC activities are significantly down-regulated after a single treatment with TPA, Chr, or OA and after multiple treatments with TPA and Chr (23, 40). Therefore, removal of the negative feedback mechanism by PKC might account, in part, for the activation of the EGFr after tumor promoter treatment; and (*c*) alteration in PTP activity may contribute to the regulation of the EGFr. Recent preliminary studies

(41) show that there is substantial PTP activity towards the EGFr in cytosol and particulate fractions from mouse epidermis. The identity and behavior of PTP(s) during tumor promotion require further investigation. Although at present the exact mechanism(s) for EGFr activation is undefined, one or a combination of several of the above mechanisms may contribute to the overall activation of the EGFr in tumor promoter-treated mouse skin.

As part of the present study, we also examined several downstream substrates for the EGFr. As shown in Figs. 1 and 4, several protein bands in the molecular weight range of M_r ~80,000–85,000 appeared to have elevated phosphotyrosine content in tumor promoter-treated epidermis. In addition, PLCγ1 (M_r 150,000) was examined as a candidate downstream target for the activated EGFr, since it is phosphorylated on tyrosine residues and subsequently becomes activated in many cell types in response to EGF (42, 43). Surprisingly, tyrosine phosphorylation of PLCγ1 was undetectable in plasma membrane preparations (Fig. 1) and *in vivo* samples following immunoprecipitation (Fig. 2). Furthermore, we failed to detect tyrosine phosphorylation of PLCγ1 in lysates from EGF-stimulated keratinocytes (Fig. 3), and in membrane preparations stimulated with EGF *in vitro* (data not shown) by Western blot analysis. Similar observations were reported by Reynolds *et al.* (44) in cultured normal human skin fibroblasts and keratinocytes in which EGFr activation was not coupled to tyrosine phosphorylation of PLCγ1. In contrast, a recent study using newborn mouse primary keratinocytes stimulated with TGF-α reported tyrosine phosphorylation of PLCγ1 (45). At the present time, we cannot exclude the possibility that activation of the EGFr led to tyrosine phosphorylation of PLCγ1, but at a time not monitored in our current studies. PI3-K, another downstream target of activated EGFr and ezrin, an important substrate for the EGFr, was present in our epidermal membrane preparations (Fig. 1). Elevated phosphotyrosine was detected in protein bands with molecular weights corresponding to PI3-K and ezrin in preparations from tumor promoter-treated mice (Fig. 1). Further confirmation that these phosphoproteins are PI3-K and ezrin, respectively, must await additional experiments.

In conclusion, the present study has demonstrated that the EGFr is activated in tumor promoter-treated mouse epidermis and in mouse skin tumors. Also, two tyrosine kinase inhibitors having some specificity toward the EGFr blocked TPA-induced hyperplasia and EGFr activation in mouse epidermis. These results provide strong evidence for an active role of EGFr tyrosine kinase in the proliferative effect of tumor promoters. Our findings, in conjunction with other findings on TGF-α overexpression in tumor promoter-treated epidermis, support the conclusion that signaling through the EGFr pathway plays an important role in the tumor promotion stage of multistage carcinogenesis in mouse skin.

Materials and Methods

Reagents

OA and TPA were obtained from LC Services (Woburn, WA). Chr was purchased from ICN Pharmaceuticals, Inc. (Plainview, NY) and purified as described previously (46). Anti-phosphotyrosine antibody (PY20) was purchased from either ICN (Costa Mesa, CA) or Transduction Laboratories (Lexington, KY). EGF, anti-PLCγ1, and anti-PI3-K antibodies were obtained from UBI (Lake Placid, NY). Anti-ezrin anti-

body was from Transduction Laboratories, and protein A-agarose was from Oncogene Science, Inc. (Cambridge, MA). Anti-EGFr polyclonal antibody RK2 was prepared as described (47). Anti-EGFr polyclonal antibody 1382 was a generous gift from Dr. H. Shelton Earp (Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, NC). Tyrphostins (RG13022 and RG50864) were provided by Rhone-Poulenc Rorer (Collegeville, PA).

Animals and Treatments

Female SENCAR mice were obtained from the National Cancer Institute (Frederick, MD) and used when 7–9 wk of age. Prior to treatment, mice were shaved on the dorsal side with surgical clippers. Mice were allowed to rest for 2 days prior to use. TPA (3.4 nmol), Chr (220 nmol), OA (2.5 nmol), or acetone (0.2 ml) was topically applied to the shaved dorsal skin. For these experiments, mice received multiple treatments according to regimens (TPA and OA, twice a week; Chr, once a week) that yield maximal responses for both epidermal hyperplasia and skin tumor promotion by these compounds (48–50).

Cell Culture and EGF Stimulation

Epidermal basal cells from SENCAR mice were isolated and cultured in MEM-2 medium with 1% FBS-0.04% penicillin-streptomycin solution and a Ca^{2+} concentration of 0.04 mM as described previously (51). Cells were plated at a density of 10^7 cells/100-mm tissue culture dish. Cells were starved for 24 h in 0.5% BSA/MEM-2 in the absence of all growth factors at day 3. After two washes with the starvation medium, cells were stimulated with 40 ng/ml EGF for 2–30 min and lysed with ice-cold lysis buffer [1% Triton X-100, 10% glycerol, 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM PMSF, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 1 mM Na_3VO_4, 50 mM NaF, 10 mM sodium PP_i, 10 mM p-nitrophenyl phosphate, and 50 µM sodium molybdate]. Cell lysates were further homogenized with a Polytron homogenizer and centrifuged at 12,000 × g for 15 min at 4°C. One hundred µl of the lysate was denatured in 2× SDS sample buffer and stored at –70°C. The rest of the supernatant was aliquoted and stored at –70°C or used for immunoprecipitation immediately.

In Vivo Sample Preparation

Plasma Membranes. After various tropical treatments, SENCAR mice were killed by cervical dislocation. The dorsal skins were treated with a depilatory agent (≤1 min), followed by washing. The skin was excised, and epidermal tissue was scraped off with a razor blade into homogenization buffer [200 mM sucrose, 25 mM HEPES (pH 7.4), 5 mM EGTA, 50 mM NaF, 50 µM Na_3VO_4, 1 mM PMSF, and 10 µg/ml leupeptin]) and homogenized with a Polytron PT10 homogenizer (10-s bursts at setting 6 three times). The epidermal homogenate was centrifuged at 800 × g for 10 min. The 800 × g supernatant was layered on a 35% sucrose cushion. Plasma membrane was collected from the interface after centrifugation at 100,000 × g for 30 min. The membrane preparations were washed twice with 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM sodium PP_i, 5 mM Na_3VO_4, 10 mM p-nitrophenyl phosphate, 20 µg/ml leupeptin, 10 µg/ml

pepstatin A, and 1 mM PMSF and used directly or stored at –70°C for short time periods prior to use.

Microsomal Membranes. Treated or control dorsal mouse skins were excised and frozen in liquid nitrogen. The frozen skin pieces were ground with a mortar and pestle and homogenized in homogenization buffer. The homogenate was centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was further centrifuged at 100,000 × g for 60 min at 4°C. The microsomal fraction (i.e., pellet) was solubilized in lysis buffer.

Immunoprecipitation

Five hundred µg of membrane protein was incubated with polyclonal anti-EGFr antibody (1382) or polyclonal anti-PLCγ1 antibody for 2 h at 4°C and then incubated with protein A-agarose for another 2 h at 4°C. The immunocomplex was precipitated by brief centrifugation and washed three times with lysis buffer. Immunoprecipitates were subjected to 7% SDS-PAGE according to the method of Laemmli (52). Separated proteins were electrophoretically transferred onto nitrocellulose membranes and analyzed by immunoblotting.

Western Blot Analysis

The samples from plasma membrane preparations were electrophoresed in 7% SDS-PAGE according to the method of Laemmli (52). In all cases, electrophoresis was performed under reducing conditions. Separated proteins were electrophoretically transferred onto nitrocellulose membranes. After blocking with 1% nonfat powdered milk in TBS (0.5 M NaCl-20 mM Tris, pH 7.5), the membrane was incubated with a 1:200 dilution of anti-EGFr polyclonal antibody (RK2) and visualized by ECL (Amersham). The same method was used to detect PLCγ1, PI 3-kinase, and ezrin levels in the membrane preparation. The tyrosine phosphorylation level of the EGFr was detected using anti-phosphotyrosine antibody (PY20) and again visualized by ECL development. Relative tyrosine phosphorylation levels were normalized to relative EGFr protein levels in appropriate samples. Quantitation of the relative differences between control and treated samples was determined by densitometry using a Visage 60 (Biolmage; Millipore Corp.).

Effects of Tyrphostins on Epidermal Hyperplasia

Groups of three female SENCAR mice were treated with two applications of TPA (3.4 nmol/ mouse) given 72 h apart. Tyrphostin (40 µg in 0.2 ml acetone) was topically applied 10 min before and 12, 24, and 36 h after each TPA treatment. Controls received acetone (0.2 ml) in place of the tyrphostin or TPA. Forty-eight h after the last TPA treatment, mice were sacrificed, and the skin was excised for histological evaluation. For determination of labeling index, mice received an i.p. injection of [^{3}H]thymidine (2 µCi/g body weight) 30 min prior to sacrifice. Skin sections were processed for conventional hematoxylin and eosin staining or autoradiography. Sections coated with nuclear tack emulsion were exposed for 18 days, then developed and stained with hematoxylin and eosin. For the quantitative determinations of the labeling index, only cells with five grains or more on the nucleus were considered labeled. A minimum of 1000 interfollicular epidermal basal cells was observed for calculation of the labeling index. The measurements of the epidermal thickness (except the horny

layer) were performed using an Olympus ocular micrometer at objective lens magnifications of ×40. At least 12 areas of a section from each block were measured at random for determination of epidermal thickness.

Tumor Induction Experiments

Female SENCAR mice aged 7-9 weeks were shaved with surgical clippers, and only those mice in the resting phase of the hair growth cycle were used. For the production of skin tumors, mice were initiated with DMBA (10 nmol), followed 2 weeks later by twice-weekly dorsal applications of TPA (3.4 nmol). Papillomas were harvested after 18 weeks of promotion. Tumors were quickly removed with surgical scissors, trimmed to remove any normal or necrotic tissue, snap frozen in liquid nitrogen, and stored at −70°C prior to use for membrane preparation.

Acknowledgments

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Involvement of PYK2 in Angiotensin II Signaling of Vascular Smooth Muscle
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Involvement of PYK2 in Angiotensin II Signaling of Vascular Smooth Muscle Cells

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Abstract—PYK2, a recently identified Ca^{2+}-sensitive tyrosine kinase, has been implicated in extracellular signal-regulated kinase (ERK) activation via several G protein–coupled receptors. We have reported that angiotensin II (Ang II) induces Ca^{2+}-dependent transactivation of the epidermal growth factor receptor (EGFR) which serves as a scaffold for preactivated c-Src and downstream adaptors (Shc/Grb2), leading to ERK activation in cultured rat vascular smooth muscle cells (VSMC). Herein we demonstrate the involvement of PYK2 in this cascade. Ang II rapidly induced tyrosine phosphorylation of PYK2, whose effect was completely inhibited by an AT_1 receptor antagonist and an intracellular Ca^{2+} chelator. A Ca^{2+} ionophore also induced PYK2 tyrosine phosphorylation to a level comparable with that by Ang II, whereas phorbol ester–induced phosphorylation was less than that by Ang II. Moreover, PYK2 formed a complex coprecipitable with catalytically active c-Src after Ang II stimulation. Although a selective EGFR kinase inhibitor completely abolished Ang II–induced recruitment of Grb2 to EGFR and markedly attenuated Ang II–induced ERK activation, it had no effect on Ang II–induced PYK2 tyrosine phosphorylation or its association with c-Src and Grb2. These data suggest that the AT_1 receptor uses Ca^{2+}-dependent PYK2 to activate c-Src, thereby leading to EGFR transactivation, which preponderantly recruits Grb2 in rat VSMC. (*Hypertension.* 1999;33[part II]:201-206.)

Key Words: angiotensin II ■ receptors, angiotensin ■ proline-rich tyrosine kinase 2 ■ c-Src
■ epidermal growth factors ■ muscle, smooth, vascular ■ signal transduction

Angiotensin II (Ang II), a dominant hemodynamic effector of the renin-angiotensin system, has been shown to promote hypertrophy or hyperplasia, or both, of vascular smooth muscle cells (VSMC),[1–3] cardiac myocytes[4] and cardiac fibroblasts.[5] Ang II also enhances migration and extracellular matrix production of VSMC.[6] Therefore, it is now widely believed that Ang II plays a key role in cardiovascular remodeling associated with hypertension, atherosclerosis, restenosis after vascular injury, heart failure, and even diabetes. This notion is supported by results of numerous in vivo experiments, as well as recent clinical trials, demonstrating multiple beneficial effects of ACE inhibitors and angiotensin type 1 receptor (AT_1R) antagonists in these disease states.[7,8]

Thus, much progress has recently made to elucidate the signal transduction mechanisms leading to the growth-promoting effect through a G protein–coupled receptor (GPCR), AT_1R. It provides an exciting aspect that AT_1R shares typical signaling events with growth factor receptor such as tyrosine kinase activation and subsequent phosphorylation of the specific substrates accompanied by selective

protein/protein interaction, resulting in activation of extracellular signal-regulated kinases (ERKs).[6,9] We recently reported that Ang II induces Ca^{2+}-dependent transactivation of the epidermal growth factor receptor (EGFR) that serves as a scaffold for preactivated c-Src kinase and downstream adaptor proteins, Shc/Grb2, leading to $p21^{ras}$/ERK activation in cultured rat VSMC.[10] However, the mechanism linking AT_1R to the receptor tyrosine kinase EGFR has not been clear.

Recently, a novel nonreceptor tyrosine kinase with a high sequence homology to p125 focal adhesion kinase (FAK) was cloned by several groups and named proline-rich tyrosine kinase 2 (PYK2),[11] cell adhesion kinase β,[12] related adhesion focal tyrosine kinase,[13] and calcium-dependent tyrosine kinase.[14] In PC12 cells, PYK2 mediates the recruitment of Grb2/Sos and subsequent $p21^{ras}$-dependent ERK activation in response to intracellular Ca^{2+} accumulation by a GPCR agonist, bradykinin, as well as membrane depolarization.[11] Moreover, PYK2 seems to operate these process in concert with c-Src.[15] Recently, Ang II has also been shown to activate PYK2 in liver epithelial cells.[14] The common feature that both EGFR and PYK2 signaling by GPCRs require intracel-

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lular Ca^{2+} elevation and c-Src activation prompted us to examine the possible involvement of PYK2 in the growth-promoting signal by Ang II in VSMC. In the present study, we assessed the contribution of PYK2 to the tyrosine kinase cascade operated through AT_1R that might exist upstream of the ERK activation in VSMC.

Methods

Materials

Ang II and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma. Recombinant human EGF was from Upstate Biotechnology. AG1478, A23187, and BAPTA-AM were from Calbiochem. An agarose-conjugated glutathione-S-transferase (GST)-Grb2 fusion protein and protein A/G-agarose were from Santa Cruz Biotechnology. CV11974 was a generous gift of Takeda Pharmaceutical Co. Anti-PYK2 polyclonal antibody (pAb) (06–559) and anti-phosphotyrosine monoclonal antibody (mAb) (4G10) were obtained from Upstate Biotechnology. Anti-PYK2 mAb (P47120) was from Transduction Laboratories. Anti-Src pAb (SRC2) and anti-EGF receptor pAb (1005) were from Santa Cruz Biotechnology. Anti-Src mAb (clone 327) was from Calbiochem. The mAb directed to Tyr530-dephosphorylated c-Src (clone 28) was prepared as described previously and selectively recognizes the active form of c-Src.[16] Horseradish peroxidase-conjugated second antibodies were from Amersham.

Cell Culture

VSMC were prepared from the thoracic aorta of 12-week-old Sprague-Dawley rats (Charles River Breeding Laboratories) by the explant method and cultured in Dulbecco's modified Eagle's medium containing 10% FCS, penicillin, and streptomycin as previously described.[17] Subcultured VSMC from passages 3 through 15 were used in the experiments. The predominant expression of AT_1R, but not of AT_2R, was confirmed by the binding study.[18] Subconfluent cells were made quiescent under serum-free condition for 3 days.

Immunoprecipitation and Immunoblotting

Cells were lysed by adding ice-cold lysis buffer, pH 7.5, containing 50 mmol/L HEPES, 50 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 1 mmol/L Na_3VO_4, 100 mmol/L NaF, 30 mmol/L 2-(p-nitrophenyl)phosphate, 1 mmol/L PMSF, 10 mg/mL leupeptin, and 10 mg/mL aprotinin and centrifuged for 5 minutes at 14 000g. Supernatant was mixed with the antibodies for immunoprecipitation and rocked at 4°C for 2 to 16 hours, and then protein A/G Sepharose was added and incubated for an additional 2 to 16 hours. Immunoprecipitates were washed in lysis buffer, solubilized in Laemmli's sample buffer with 2-mercaptoethanol, resolved by SDS-PAGE, and transferred to nitrocellulose membrane. After blocking with 5% milk, the membrane was treated with a primary antibody, followed by a secondary antibody conjugated with horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham) as described.[10] For immunoblot analysis of Grb2-associable proteins, agarose-conjugated GST-Grb2 fusion protein was rocked with Triton X-100–treated cell lysate at 4°C for 2 to 16 hours and washed with lysis buffer. Bound proteins were solubilized, resolved by SDS-PAGE, and subjected to immunoblotting as described.

Results

Ang II Activates PYK2 Through AT_1R

To assess whether Ang II activates PYK2 in VSMC, the effect of Ang II on phosphotyrosine content of PYK2 was examined. Treatment of quiescent rat VSMC with Ang II (10^{-7} mol/L) markedly increased tyrosine-phosphorylated PYK2 as early as 2 minutes; neither phosphorylated band nor

A Ang II (100 nM)

B IP: IgG PYK2
basal Ang II

IP: PYK2
IB: pTyr
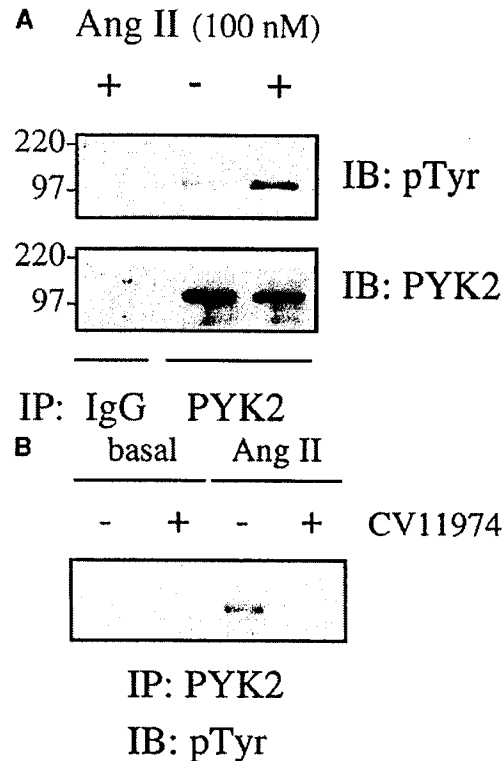
Figure 1. Ang II stimulates tyrosine phosphorylation of PYK2 through AT_1R. A, VSMC were stimulated with Ang II (10^{-7} mol/L) for 2 minutes. After cell lysis, immunoprecipitation (IP) was performed with anti-PYK2 pAb or normal rabbit IgG. Precipitates were analyzed by immunoblotting (IB) with anti-phosphotyrosine (pTyr) mAb and anti-PYK2 mAb. B, VSMC pretreated with or without CV11974 (10^{-5} mol/L) for 30 minutes and then stimulated with Ang II (10^{-7} mol/L) for 2 minutes were subjected to IP and IB.

immunoprecipitated PYK2 was observed when normal rabbit IgG was used for the immunoprecipitation (Figure 1A). Pretreatment with 10^{-5} mol/L CV11974, a selective AT_1R antagonist, completely blocked Ang II–induced tyrosine phosphorylation of PYK2 (Figure 1B). These data indicate that Ang II activates PYK2 via AT_1R in rat VSMC.

Calcium-Dependent PYK2 Activation by Ang II

PYK2 activation through GPCRs involves intracellular Ca^{2+} elevation and/or protein kinase C (PKC) activation in PC12 cells.[11] Stimulation of AT_1R activates phospholipase $C\beta$ to increase cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and activate PKC in VSMC.[6] However, both $p21^{ras}$/ERK activation[18] and EGFR transactivation[10] via AT_1R are mainly mediated by an increase in $[Ca^{2+}]_i$. To determine the Ca^{2+} dependence of PYK2 activation by Ang II in VSMC, the effect of an intracellular Ca^{2+} chelator (BAPTA-AM) was examined. Pretreatment with 10^{-5} mol/L BAPTA-AM, but not with its solvent DMSO (0.1%), completely inhibited the Ang II–induced PYK2 phosphorylation (Figure 2A). The Ca^{2+} ionophore A23187 (10^{-5} mol/L) also induced PYK2 tyrosine-phosphorylation comparable with that by 10^{-7} mol/L Ang II, whereas a PKC activator, PMA (10^{-6} mol/L), minimally induced PYK2 phosphorylation (Figure 2B). These data demonstrated that Ang II–induced PYK2 activation requires

A DMSO   BAPTA

\- + - +   Ang II

IB: pTyr

IB: PYK2

B

IP: PYK2

basal   Ang II   A23187   PMA

IP: PYK2

IB: pTyr

Figure 2. Calcium-dependent PYK2 tyrosine phosphorylation by Ang II. A, VSMC were pretreated with 10^{-5} mol/L BAPTA-AM (BAPTA) or 0.1% dimethylsulfoxide (DMSO) for 30 minutes and then stimulated with Ang II (10^{-7} mol/L) for 2 minutes. Cell lysates were immunoprecipitated (IP) with anti-PYK2 pAb, followed by immunoblotting (IB) with anti-phosphotyrosine (pTyr) mAb and anti-PYK2 mAb. B, VSMC stimulated with Ang II (10^{-7} mol/L), A23187 (10^{-5} mol/L), or PMA (10^{-6} mol/L) for 2 minutes were subjected to IP and IB.

an increase in $[Ca^{2+}]_i$ rather than activation of PKC in rat VSMC.

Association of PYK2 With c-Src by Ang II

The autophosphorylation of PYK2 at Tyr402 with the conserved YAEI sequence provides a selective binding site for the SH2 domains of Src family tyrosine kinase for its activation, which is essential for the PYK2-mediated ERK activation by several GPCR agonists.[15] We[10] and others[19] have recently demonstrated that c-Src is involved in the Ang II–induced ERK activation in rat VSMC. To determine whether c-Src plays a role in PYK2 signaling activated by Ang II in VSMC, PYK2 immunoprecipitates after Ang II treatment were analyzed by immunoblotting with antibodies against active c-Src and phosphotyrosine. Ang II (10^{-7} mol/L) initiated tyrosine phosphorylation of PYK2 as early as in 1 minute, which was sustained up to 5 minutes, with concomitant transient (1 to 2 minutes) association of PYK2 with catalytically active c-Src (Figure 3A). A $\approx$120-kDa tyrosine-phosphorylated protein was induced to associate with c-Src by Ang II, which comigrated with the band detected by the anti-PYK2 antibody (Figure 3B). A23187 (10^{-5} mol/L) also increased the association of PYK2 with active c-Src (data not shown). These data provide evidence

A Ang II (min)

0   1   2   5

IB: pTyr

IB:PYK2

IB: c-Src (clone 28)

IP: PYK2

B   Ang II

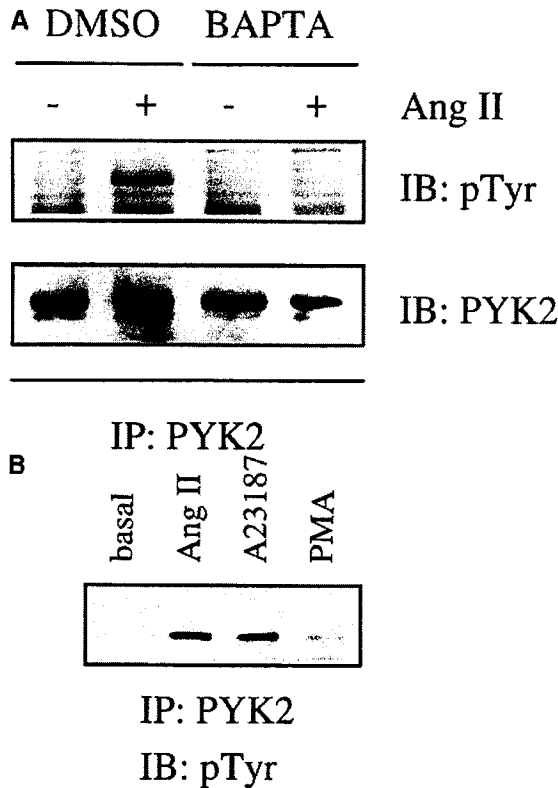\-   +

139–

80–   IB:PYK2

139–

80–   IB: pTyr

IB: c-Src

IP: c-Src
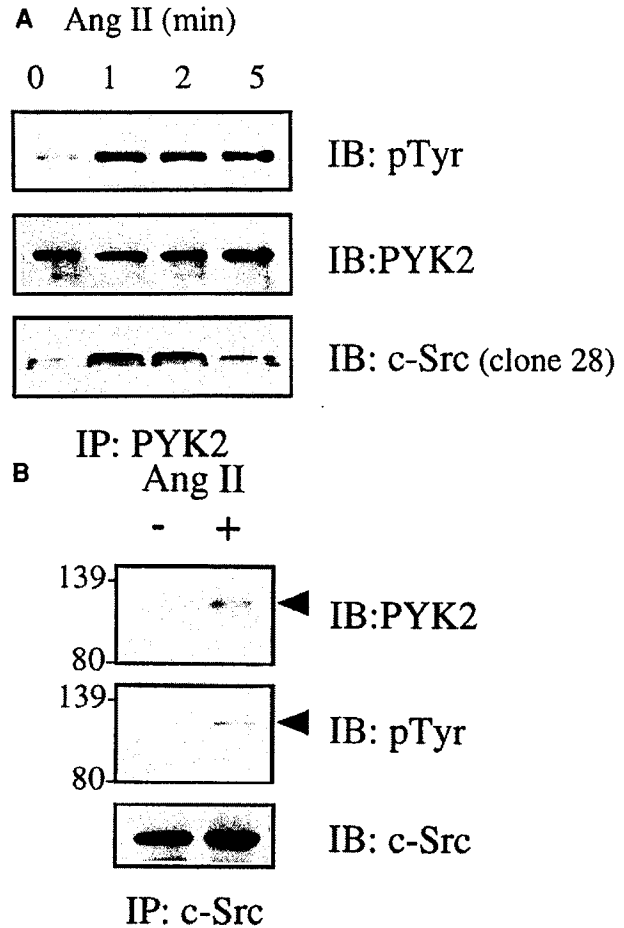
Figure 3. Association of PYK2 with c-Src on Ang II stimulation. A, VSMC were stimulated with Ang II (10^{-7} mol/L) for indicated durations. Cell lysates were immunoprecipitated (IP) with anti-PYK2 pAb, followed by immunoblotting (IB) with anti-phosphotyrosine (pTyr) mAb, anti-PYK2 mAb, and anti-c-Src mAb (clone 28) by repeated reprobing. B, VSMC were stimulated with Ang II (10^{-7} mol/L) for 2 minutes. Cell lysates were immunoprecipitated (IP) with anti-c-Src pAb (SRC2), followed by immunoblotting (IB) with anti-PYK2 mAb, anti-phosphotyrosine (pTyr) mAb, and anti-c-Src mAb (clone 327) by repeated reprobing. Arrowheads indicate the position of PYK2.

for the involvement of c-Src in PYK2 signaling initiated by AT$_1$R, presumably through an increase in $[Ca^{2+}]_i$, a new finding to our knowledge.

Effect of EGFR Inhibition of PYK2 Signaling

We have recently shown that c-Src exists upstream of EGFR transactivation, which plays an essential role in the AT$_1$R-mediated Ca^{2+}-dependent ERK activation in rat VSMC.[10] Thus, it could be hypothesized that PYK2 may contribute to the EGFR transactivation through c-Src. To elucidate the hierarchical order of PYK2, c-Src, and EGFR, the effect was studied of a selective EGFR kinase inhibitor, AG1478, on the PYK2 phosphorylation and its association with c-Src. Neither phosphorylation of PYK2 nor its association with c-Src by Ang II (10^{-7} mol/L) was inhibited by AG1478 at 2.5×10^{-7} mol/L (Figure 4A), a concentration effective in inhibiting Ang II–induced ERK activation in rat VSMC.[10] Furthermore, EGF (100 ng/mL) did not affect the phosphotyrosine content of PYK2 in rat VSMC (data not shown).

A DMSO AG1478

‒ + ‒ + Ang II (100 nM)

[gel band] IB: pTyr

[gel band] IB:PYK2

[gel band] IB: c-Src (clone 28)

IP: PYK2

B DMSO AG1478

‒ + ‒ + Ang II (100 nM)

[gel band] IB: EGFR

[gel band] IB:PYK2

GST-Grb2-FP

Figure 4. Effects of AG1478 on Ang II–induced association of PYK2 with c-Src and Grb2. A, VSMC were pretreated with 2.5×10^{-7} mol/L AG1478 or 0.1% dimethylsulfoxide (DMSO) for 30 minutes and then stimulated with Ang II (10^{-7} mol/L) for 2 minutes. Cell lysates were immunoprecipitated (IP) with anti-PYK2 pAb, followed by immunoblotting (IB) with anti-phosphotyrosine (pTyr) mAb, anti-PYK2 mAb, and anti-c-Src mAb (clone 28). B, VSMC were pretreated with 2.5×10^{-7} mol/L AG1478 or 0.1% dimethylsulfoxide (DMSO) for 30 minutes and then stimulated with Ang II (10^{-7} mol/L) for 2 minutes. After cell lysis, GST-Grb2 fusion protein was added. Proteins associated with the fusion protein were subjected to immunoblotting (IB) with anti-PYK2 mAb and anti-EGFR pAb.
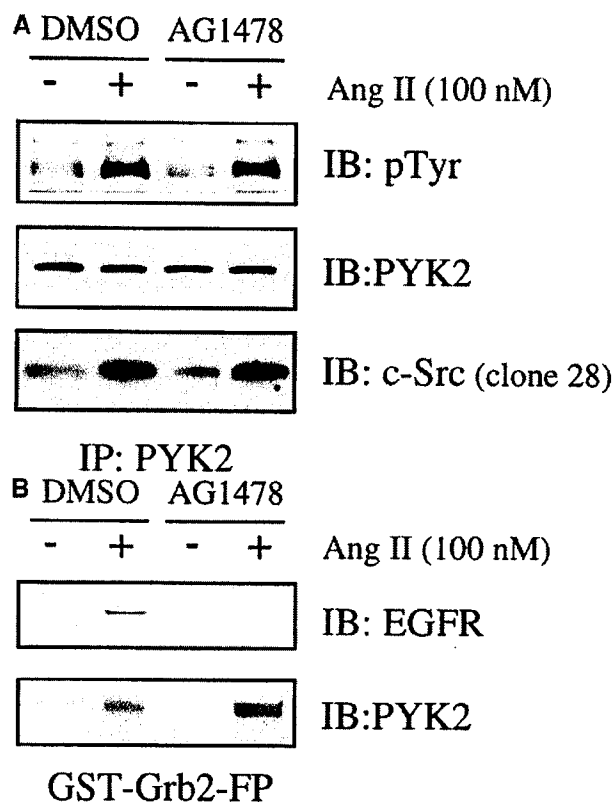
The activated PYK2 has been shown to recruit Grb2 for the ERK activation in neuronal cells.[11] To elucidate whether similar mechanism is operated in VSMC after stimulation with Ang II, the lysates of VSMC stimulated by Ang II (10^{-7} mol/L) with or without pretreatment of AG1478 (2.5×10^{-7} mol/L) for 30 minutes were coprecipitated with GST-Grb2-fusion protein, followed by immunoblotting with antibodies against EGFR or PYK2. Ang II increased the amounts of PYK2 coprecipitable with the fusion protein regardless of the presence of AG1478, whereas AG1478 completely inhibited Ang II–induced association of EGFR with the fusion protein (Figure 4B). Thus, activation of PYK2, as well as its association with c-Src and Grb2 in response to Ang II, occurs independent of EGFR kinase activity, suggesting that PYK2 may be located upstream of and/or in parallel with the EGFR in VSMC.

Discussion

A growing body of evidence indicates that the growth-promoting effect by Ang II is mediated by activation of several protein tyrosine kinases.[6,9] Earlier studies from our laboratory indicated that a Ca^{2+}-dependent tyrosine kinase or kinases may transmit AT_1R signal to the ERK cascade in rat VSMC.[18] Subsequently, we have shown EGFR as such a tyrosine kinase.[10] Here, we further identified and characterized another AT_1R-responsive Ca^{2+}-dependent tyrosine kinase as PYK2 in rat VSMC.

PYK2 has been shown to be regulated by Ca^{2+} signal in PC12 cells[11] and constitutes a major Ca^{2+}-dependent tyrosine kinase in Ang II–stimulated liver epithelial cells.[14] The Ca^{2+}-dependency of the Ang II–induced PYK2 activation as demonstrated in this study is consistent with a recent report showing that Ang II– and platelet-derived growth factor–stimulated PYK2 activation was inhibitable with the intracellular Ca^{2+} chelator in rat VSMC,[20] whereas the importance of PKC was also suggested. However, the present results appear to demonstrate that PYK2 phosphorylation by PMA is much weaker than those by Ang II and a Ca^{2+} ionophore, suggesting a preferential role of calcium to PKC in regulation of PYK2 in VSMC. Because PYK2, which lacks calmodulin-binding motif, cannot be activated by either Ca^{2+} or calmodulin in vitro,[11] the mechanism by which Ca^{2+} signal activates PYK2 remains to be determined.

Src family tyrosine kinase has been implicated in the ERK activation by various agonists for GPCRs, including AT_1R.[21–23] Recently, it has been reported that both G_q and G_i agonists, such as bradykinin and lysophosphatidic acid, respectively, induced association of PYK2 with c-Src through binding of autophosphorylated Tyr402 of PYK2 to the SH2 domain of c-Src, thereby leading to c-Src activation.[15] The activated c-Src could further phosphorylate PYK2 at Tyr881 followed by the LNV sequence and an adaptor protein Shc, thereby recruiting the Grb2/Sos complex. These events are believed to be essential for the ERK activation by GPCR agonists in PC12 cells.[15] The calcium-dependent PYK2/c-Src activation has also been shown to bridge both G_i- and G_q-coupled receptors to the ERK activation in HEK 293 cells.[24] In rat VSMC, we have recently shown that Ang II increased transient association of active c-Src with Shc that is contingent on Shc phosphorylation.[10] In the present study, we further demonstrated that PYK2 formed a complex with an active c-Src and Grb2 on Ang II stimulation. Therefore, it is reasonable to speculate that PYK2 may contribute to the Ang II–induced ERK activation in concert with Src family tyrosine kinase and adaptors (Shc and Grb2) in cells where AT_1R promotes cell growth, such as in VSMC.

In addition to PYK2 and c-Src, combination of multiple tyrosine kinases appears to be involved in the ERK activation by GPCR agonists depending on cell type. For example, the G_q-coupled ERK activation requires Csk, Lyn, and Syk, whereas the G_i-coupled activation requires Btk and Syk in avian lymphoma cells.[25] We and others have recently shown that c-Src acts upstream of EGFR transactivation to feed into the ERK cascade through G_q-coupled AT_1R in rat VSMC[10] and G_i-coupled lysophosphatidic acid and α_{2A}-adrenergic receptors in COS-7 cells,[26] respectively. Interestingly, not only PYK2 and c-Src,[15] but also EGFR,[27] appear to be essential for the Ca^{2+}-dependent ERK activation by GPCR agonists in PC12 cells. Thus, the Ang II–induced Ca^{2+}-
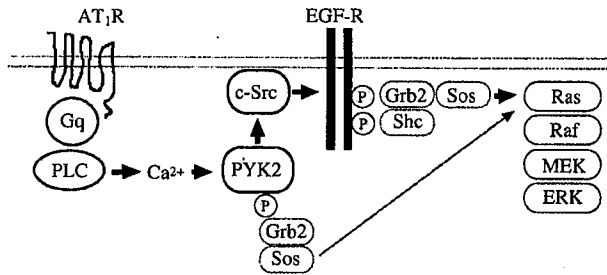
Figure 5. Possible involvement of PYK2 in signal transduction of VSMC operated through AT₁R. Ang II induces ERK cascade (Ras, Raf, MEK, ERK) through adaptors (Shc and Grb2) and Sos recruited through EGFR that is transphosphorylated by c-Src. The c-Src activation is mainly mediated through PYK2, which senses intracellular Ca^{2+} elevation by G_q/phospholipase C–coupled AT₁R. PYK2 may also contributes to the direct recruitment of Grb2/Sos complex for ERK activation, although possibly constituting a minor component.

dependent PYK2 activation accompanied by its interaction with c-Src as demonstrated in this study and the Ang II–induced association of c-Src with EGFR as demonstrated in our previous study[10] strongly suggest that PYK2 function is mainly located upstream of the AT₁R-mediated EGFR transactivation. This is consistent with the present observation that neither PYK2 phosphorylation nor its association with active c-Src requires EGFR kinase activity after Ang II stimulation.

Alternatively, PYK2 could function in parallel with EGFR to feed into the ERK cascade because Ang II–induced association of Grb2 with PYK2 occurred even when the association of Grb2 with EGFR was completely blocked by AG1478. In liver epithelial cells, Ca^{2+}- and PKC-dependent PYK2 activation by Ang II was reported,[14] whereas the Ang II–induced EGFR transactivation appeared to be driven only when cellular PKC was depleted.[28] However, we have recently shown that AG1478 markedly inhibited the Ang II–induced ERK activation.[10] Taken together, we submit that the recruitment of Grb2 by PYK2 contributes little, if any, to the ERK activation through AT₁R and that the ERK activation by Ang II appears to be preferentially mediated by the recruitment of Grb2 to the EGFR in VSMC. A possible involvement of PYK2 in signal transduction of Ang II–induced ERK activation is illustrated in Figure 5.

PYK2 may account for other signaling pathways than the ERK cascade by AT₁R in VSMC. PYK2 is involved in c-Jun amino-terminal kinase (JNK) activation induced by tumor necrosis factor-α, ultraviolet irradiation, and osmotic shock.[29] It has been shown that PYK2 activation is correlated with JNK activation[14] and p70 ribosomal S6 kinase activation, but not ERK activation,[30] in Ang II–stimulated rat liver epithelial cells. In addition, PYK2 has a "focal adhesion-targeting domain" homologous to that of FAK.[13] In fact, PYK2 has been shown to be tyrosine-phosphorylated after β₁-integrin stimulation[31] and to be associated with a cytoskeletal protein, paxillin.[32] In this regard, it has recently been reported that paxillin is tyrosine-phosphorylated by and associates with PYK2 in Ang II–stimulated rat liver epithelial cells.[33] Because these tyrosine kinases (PYK2, c-Src, EGFR) may phosphorylate each other as well as respective specific

substrates and recruit additional signaling molecules, several signaling events branching at the level of these kinases will account for diverse functions of the AT₁R in a tissue- and cell type–specific manner.

In conclusion, we have demonstrated that Ang II induces a Ca^{2+}-dependent PYK2 activation and its interaction with c-Src and Grb2 in rat VSMC. Further elucidation of cross-talk between AT₁R and protein tyrosine kinases, as well as their downstream signals, should unravel the exact role of Ang II in the mechanism of vascular remodeling under pathological states, such as in hypertension, atherosclerosis, and restenosis after angioplasty.

Acknowledgments

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Stimulation of growth factor receptor signal transduction by activation of voltage-sensitive calcium channels

(epidermal growth factor receptor/Ras/mitogen-activated protein kinase)

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ABSTRACT   To understand the mechanisms by which electrical activity may generate long-term responses in the nervous system, we examined how activation of voltage-sensitive calcium channels (VSCCs) can stimulate the Ras/mitogen-activated protein kinase (MAPK) signaling pathway. Calcium influx through L-type VSCCs leads to tyrosine phosphorylation of the adaptor protein Shc and its association with the adaptor protein Grb2, which is bound to the guanine nucleotide exchange factor Sos1. In response to calcium influx, Shc, Grb2, and Sos1 inducibly associate with a 180-kDa tyrosine-phosphorylated protein, which was determined to be the epidermal growth factor receptor (EGFR). Calcium influx induces tyrosine phosphorylation of the EGFR to levels that can activate the MAPK signaling pathway. Thus, ion channel activation stimulates growth factor receptor signal transduction.

Calcium influx into neurons is the critical transducer of electrical input into biochemical output (1). A wide range of neurotransmitter receptors and second messenger systems have been shown to regulate the influx of extracellular calcium through their effects on voltage-sensitive calcium channels (VSCCs) and other ion channels in a process termed neuromodulation (2–4). However, the mechanisms by which calcium influx elicits long-term neuronal responses are less clear.

Studies of the biochemical responses generated in response to calcium influx have focused on activation of cytoplasmic signaling molecules that directly bind calcium or calcium–calmodulin (CaM) complexes, such as calcium–CaM-dependent adenylate cyclases and protein kinases (5). Calcium influx also leads by indirect mechanisms to activation of the ubiquitous mitogen-activated protein kinase (MAPK) pathway (6, 7), which is a critical intermediate in long-term cellular responses such as proliferation and differentiation (8–10). Recently, we have demonstrated that stimulation of the MAPK pathway in response to calcium influx through L-type VSCCs involves activation of the small guanine nucleotide binding protein Ras (11), a protooncogene product that mediates MAPK activation in response to a wide variety of mitogens, cytokines, and trophic factors such as nerve growth factor (NGF) (8–10). To understand the mechanisms by which electrical activity may initiate long-term responses in the nervous system, we examined how calcium influx leads to activation of Ras.

Growth factors initiate signaling processes that lead to Ras activation by binding to transmembrane receptors that contain intrinsic tyrosine kinase activity or, in the case of cytokine receptors, that are associated through their cytoplasmic domains with nontransmembrane protein tyrosine kinases such as the Src family members (12–14). Ligand binding induces receptor dimerization and autophosphorylation on tyrosine residues. These phosphorylated tyrosines create binding sites for Src homology 2 (SH2) domains, which are present in a number of different signaling molecules that associate with activated growth factor receptors (15, 16). SH2 domains bind to phosphorylated tyrosine residues and adjacent amino acid sequences, which determine the specificity of the interaction.

One class of signaling molecule that inducibly binds to growth factor receptors is the adaptor protein, which lacks catalytic moieties but mediates protein–protein interactions via modular domains such as SH2 domains. One of the adaptor proteins that inducibly associates with tyrosine-phosphorylated growth factor and cytokine receptors is the SH2/collagen protein (Shc) (17). Shc is itself also inducibly phosphorylated on tyrosine in response to growth factor and cytokine stimulation (18–22), which creates a consensus binding site (pYXN) that is recognized by another SH2 domain-containing adaptor protein, growth factor receptor binding protein 2 (Grb2) (23). Grb2 contains, in addition to its SH2 domain, two SH3 domains that mediate its interaction with proline-rich sequences in the Ras guanine nucleotide exchange factor (GEF) termed mSos1 (24–28). Induction of Grb2-Sos1 association with Shc through Y317 is a potential mechanism for Ras activation in response to growth factors and cytokine stimulation (29–35).

The parallels we found previously between calcium and growth factor activation of MAPK suggested that tyrosine phosphorylation might be involved in calcium activation of Ras. We report here that calcium influx upon activation of VSCCs leads to tyrosine phosphorylation of Shc and its association with Grb2 and Sos1. In addition, this signaling complex inducibly associates with the epidermal growth factor receptor (EGFR), which is phosphorylated on tyrosine in response to calcium influx to a level that is sufficient to lead to downstream MAPK activation. Our results demonstrate that growth factor receptor signal transduction is activated in response to VSCC stimulation, which may be an important biochemical mechanism by which neuronal activity can generate long-term cellular responses.

MATERIALS AND METHODS

Materials. EGF was from Collaborative Biomedical Products (Bedford, MA), and NGF was purified from mouse salivary glands as described (36). Nifedipine was from Sigma. Anti-Trk antibodies were the generous gift of David Kaplan (67). Other antibodies were obtained from the following vendors: anti-phosphotyrosine [Tyr(p)] monoclonal antibody (mAb) 4G10 from Upstate Biotechnology (Lake Placid, NY); anti-Tyr(p) mAb PY20 from ICN; anti-Shc polyclonal antibody (pAb) and mAb from Transduction Laboratories (Lexington, KY); anti-Grb2 mAb from Upstate Biotechnology; anti-Grb2

Abbreviations: VSCC, voltage-sensitive calcium channel; CaM, calmodulin; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; GEF, guanine nucleotide exchange factor; EGF, epidermal growth factor; EGFR, EGF receptor; mAb, monoclonal antibody; pAb, polyclonal antibody.
§To whom reprint requests should be addressed.

pAb from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Sos1 pAb from Upstate Biotechnology; anti-EGFR pAb from Upstate Biotechnology; anti-ErbB2 from Oncogene Science; goat anti-mouse pAb from Calbiochem; and rabbit anti-sheep pAb from Pierce.

Cell Culture and Stimulation. PC12 cells were obtained from Simon Halegoua (68) and cultured on 100-mm tissue culture dishes (Falcon) in DMEM (GIBCO) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (GIBCO) in a humidified incubator with 10% CO_2/90% air. Cell membranes were depolarized by addition of an isosmotic solution of KCl (or NaCl control) to a final concentration of 50 mM as described (11).

Immunoprecipitation and Immunoblotting. Cells were lysed in HNTG buffer [50 mM Hepes, pH 7.5/50 mM NaCl/1% Triton X-100/10% glycerol (vol/vol)/1.5 mM $MgCl_2$/1 mM EDTA/10 mM sodium pyrophosphate/1 mM Na_3VO_4/100 mM NaF/30 mM 2-(p-nitrophenyl) phosphate/1 mM phenylmethylsulfonyl fluoride/10 µg of aprotinin per ml/10 µg of leupeptin per ml] and centrifuged at 10,000 × g for 15 min. Supernatants were mixed with primary antibody and rocked at 4°C for 1–4 h. Secondary antibody and protein A-Sepharose (Calbiochem) were added for an additional 1–2 h. Immunoprecipitates were washed three times in HNTG and resuspended in 2× Laemmli sample buffer (80 mM Tris·HCl, pH 6.8/15% glycerol/2% SDS/0.01% bromphenol blue/10% 2-mercaptoethanol). Precipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose, and analyzed by Western blotting as described (11). Antibody binding was detected by enhanced chemiluminescence (ECL; Amersham) with a secondary antibody conjugated to horseradish peroxidase. For analysis of Shc, Grb2, and Sos1 coimmunoprecipitation, 6–12% gradient gels were run, and the nitrocellulose blots were cut horizontally at the 125- and 35-kDa markers for blotting of individual proteins.

RESULTS

To examine whether tyrosine phosphorylation might be involved in calcium activation of Ras, we determined the effect of membrane depolarization on tyrosine phosphorylation of Shc and its association with Grb2 in the pheochromocytoma cell line PC12. Membrane depolarization of both NGF-differentiated and undifferentiated PC12 cells has been demonstrated to activate signaling pathways that are also activated by direct electrical stimulation of primary neurons in culture as well as by neuronal excitation *in vivo* (37). Undifferentiated PC12 cells were membrane depolarized by exposure to elevated levels of extracellular KCl to induce calcium influx through VSCCs, or cells were treated with NGF or EGF. Shc proteins were immunoprecipitated and analyzed by protein immunoblotting with antibodies to phosphotyrosine. Equal amounts of Shc were immunoprecipitated as shown by immunoblotting for Shc proteins (Fig. 1A *Bottom*). KCl induced tyrosine phosphorylation of the 48- and 56-kDa Shc isoforms, as did NGF and EGF treatment (Fig. 1A *Top*). The 65-kDa Shc isoform was inducibly tyrosine phosphorylated to a lesser extent by NGF and EGF but not by KCl. Although this may simply be due to a detection limit in the assay, the differential phosphorylation of the three Shc isoforms in response to the different stimuli may reflect specificity in the signaling pathways. These results demonstrate that membrane depolarization leads to inducible tyrosine phosphorylation of Shc, an adaptor protein that is involved in Ras activation in response to growth factor stimulation.

To determine whether KCl-induced Shc phosphorylation led to its functional association with Grb2, Shc immunoprecipitates (Fig. 1A) from PC12 cells treated with KCl, NGF, or EGF were analyzed for coprecipitation of Grb2 by immunoblotting with anti-Grb2 antibody. Membrane depolarization
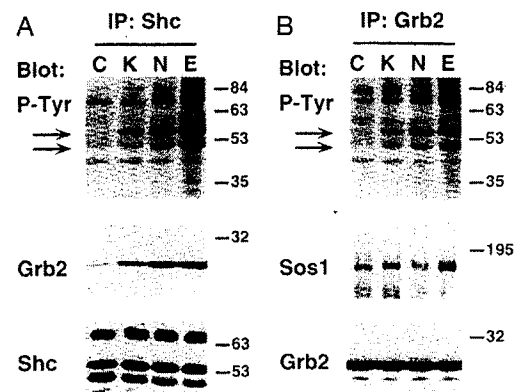

FIG. 1. Calcium induction of Shc tyrosine phosphorylation and Grb2 association. (A) PC12 cells were incubated with 50 mM NaCl control solution (lane C), 50 mM KCl (lane K), 100 ng of NGF per ml (lane N), or 10 ng of EGF per ml (lane E) for 5 min. Lysates were immunoprecipitated with anti-Shc pAb. Washed immunoprecipitates (IP) were separated by PAGE and transferred to nitrocellulose for immunoblotting with a mixture of anti-Tyr(p) antibodies 4G10 and PY20, anti-Grb2 mAb (*Middle*), or anti-Shc mAb (*Bottom*). Positions of migration of prestained molecular size markers (kDa) (Sigma) are shown. Arrows indicate phosphorylated Shc isoforms of 48 and 56 kDa. (B) PC12 cells were treated as in A. Lysates were immunoprecipitated with a pAb against Grb2, and precipitated proteins were analyzed by immunoblotting with anti-Tyr(p) antibodies (*Top*), anti-Sos1 pAb (Upstate Biotechnology; *Middle*), or anti-Grb2 mAb (*Bottom*) to confirm that equal levels of Grb2 were immunoprecipitated. Positions of migration of prestained molecular size markers are shown.

with KCl led to the inducible association of Shc with Grb2, as did NGF and EGF treatment (Fig. 1A *Middle*). This KCl-induced association of Shc with Grb2 was also demonstrated by first immunoprecipitating Grb2 and then immunoblotting with antibodies to phosphotyrosine to detect coprecipitated Shc proteins (Fig. 1B *Top*). In addition, by immunoblotting Grb2 immunoprecipitates with antibodies to Sos1, we found that Grb2 is constitutively bound to Sos1 in PC12 cells, as others have previously shown (Fig. 1B *Middle*) (38). Thus, KCl-stimulated tyrosine phosphorylation of Shc can induce its association with the Grb2-Sos1 complex.

Evidence suggests that the Ras GEF must be targeted to the plasma membrane in order to activate Ras (39). In the case of growth factor stimulation, this can be accomplished by association of the adaptor protein–GEF complexes with the receptor tyrosine kinase itself. This can occur by receptor binding directly to Grb2-Sos1 complexes through the Grb2 SH2 domain (24–28) or through binding Shc-Grb2-Sos1 complexes through the Shc SH2 domain (33–35, 40–42) or a phosphotyrosine-binding domain at the Shc N terminus (43, 44). To determine how calcium influx might target the Shc-Grb2-Sos1 signaling complex to the plasma membrane, we examined whether KCl treatment led to association of Shc, Grb2, or Sos1 with a tyrosine-phosphorylated protein that could act as a membrane anchor. Shc, Grb2, and Sos1 proteins were immunoprecipitated and analyzed for coprecipitating proteins containing phosphotyrosine by immunoblotting with anti-phosphotyrosine antibodies. KCl induced the association of an ≈180-kDa tyrosine-phosphorylated protein with Shc, Grb2, and Sos1 (Fig. 2). Surprisingly, this protein comigrated with a tyrosine-phosphorylated protein that was coprecipitated in response to EGF stimulation. A low or undetectable level of pp180 was detected in Shc, Grb2, and Sos1 immunoprecipitates from NGF-treated cells. We failed to detect coprecipitation of the NGF receptor p140trk in Shc immunoprecipitates in response to NGF, possibly because of lower endogenous levels of Trk than EGFR in these cells.

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IP: Shc   Grb2   Sos1

Blot: C K N E C K N E C K N E

P-Tyr

→   —195

—112
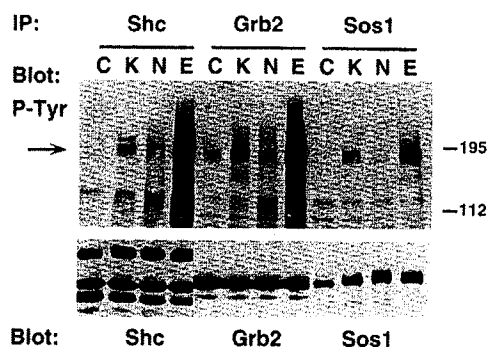
Blot: Shc   Grb2   Sos1

FIG. 2. Calcium-induced association of Shc, Grb2, and Sos1 with pp180. PC12 cells were treated with 50 mM NaCl control solution (lanes C), 50 mM KCl (lanes K), 100 ng of NGF per ml (lanes N), or 10 ng of EGF per ml (lanes E) for 5 min. Lysates were immunoprecipitated with pAb against Shc, Grb2, or Sos1, and precipitated proteins (IP) were analyzed by immunoblotting with anti-Tyr(p) antibodies (*Top*) or with antibodies to the precipitated proteins to confirm equal recovery (*Bottom*). Positions of migration of prestained molecular size markers (kDa) are shown. Arrow indicates pp180.

The EGFR can associate with Grb2 directly through binding of the Grb2 SH2 domain to phosphorylated Y1068 or Y1086 in the receptor, as well as indirectly via binding of Shc to phosphorylated Y1148 or Y1173 (41, 42, 45). The observation that a 180-kDa protein inducibly associated with Shc, Grb2, and Sos1 in response to KCl as well as EGF raised the possibility that both proteins might be the EGFR. This possibility was examined directly by immunoprecipitating Grb2 and immunoblotting with antibodies to the EGFR. Membrane depolarization with KCl induced Grb2 association with the EGFR, although to a lesser extent than treatment with 10 ng of EGF per ml (Fig. 3, lanes 1–3). This result suggests that the 180-kDa protein that is coprecipitated with Shc, Grb2, and Sos1 in response to KCl stimulation is the EGFR. Thus, calcium influx induces the association of a growth factor receptor with downstream signaling proteins that can trigger Ras activation.

To determine whether KCl-induced EGFR association with Shc, Grb2, and Sos1 was due to calcium influx through VSCCs, the effect of a specific channel antagonist on the interaction was examined. Pretreatment of PC12 cells with the L-type VSCC antagonist nifedipine or with the calcium chelator EGTA for 15 or 5 min, respectively, completely blocked the ability of KCl to induce the association of Grb2 with the

EGFR, whereas they had no effect on EGF-induced association (Fig. 3, lanes 4–9). These results demonstrate that KCl-induced association of the EGFR with Grb2 is dependent on the influx of extracellular calcium through L-type VSCCs and is not a nonspecific effect of membrane depolarization. This finding is consistent with previous observations that nifedipine and nimodipine, another dihydropyridine antagonist of L-type VSCCs, block KCl activation of Ras and that KCl activation of MAPK is blocked by nifedipine but not by ω-conotoxin, an inhibitor of N-type VSCCs (ref. 11; unpublished data). The importance of L-type VSCCs in mediating the signaling effects of membrane depolarization we observe reflects the fact that they are the primary carrier of voltage-sensitive calcium current in undifferentiated PC12 cells (46).

Since Shc and Grb2 specifically recognize tyrosine-phosphorylated proteins, the finding that these adaptor molecules inducibly associate with the EGFR upon VSCC activation suggested that calcium influx was leading to tyrosine phosphorylation of the receptor. To examine the effect of calcium influx on the phosphotyrosine content of the EGFR, the EGFR was immunoprecipitated after KCl or EGF treatment and analyzed by immunoblotting with antibodies to phosphotyrosine. Calcium influx led to inducible tyrosine phosphorylation of the EGFR within 20 sec of membrane depolarization (Fig. 4 *Top*). This calcium-induced phosphorylation of the EGFR can therefore account for the inducible association of the receptor with Shc–Grb2–Sos1 in response to membrane depolarization.

To determine whether calcium-induced EGFR tyrosine phosphorylation was likely to lead to physiologically meaningful receptor responses, we titrated down the dose of EGF to a level (1 ng/ml) that produced an induction of EGFR tyrosine phosphorylation comparable to that induced by KCl (Fig. 4 *Top*). We then examined whether this level of EGFR phosphorylation was sufficient to produce downstream responses to EGF. MAPK activation was monitored as an indicator of physiologically important signal transduction, since MAPK activation has been demonstrated to be critical for a variety of long-term cellular responses to extracellular stimuli, including cell proliferation and differentiation (8–10). Both membrane depolarization and treatment with EGF (1 ng/ml) led to inducible tyrosine phosphorylation of the 42- and 44-kDa isoforms of MAPK. The identification of these tyrosine phosphorylated bands as activated MAPK was confirmed by immunoblotting with antibodies that specifically recognize the

IP: Grb2

Ctl   Nif   EGTA

C K E C K E C K E

Blot:

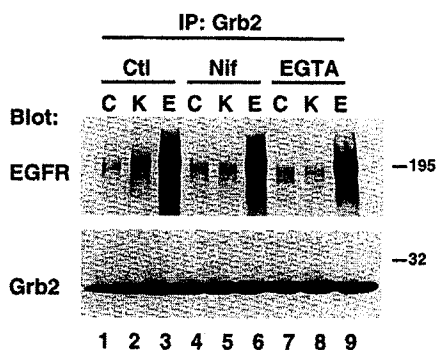EGFR   —195

Grb2   —32

1 2 3 4 5 6 7 8 9

FIG. 3. Calcium induction of Grb2 association with the EGFR. PC12 cells were treated with 50 mM NaCl control solution (lanes C), 50 mM KCl (lanes K), or 10 ng of EGF per ml (lanes E) for 2 min. Lysates were immunoprecipitated with anti-Grb2 pAb, and precipitated proteins (IP) were analyzed by immunoblotting with anti-EGFR pAb (*Top*) or anti-Grb2 mAb (*Bottom*). Cells were pretreated as follows: lanes 1–3, vehicle control for 15 min (Ctl); lanes 4–6, 5 μM nifedipine for 15 min (Nif); lanes 7–9, 3 mM EGTA for 5 min (EGTA). Positions of migration of prestained molecular size markers (kDa) are shown.

KCl   EGF

Time (min): 0 .33 1 2.5 5 10 30 60 0 .33 1 2.5 5 10 30 60

IP: EGFR   —195

Lysates   —53

→
→   —35
    —32

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Blots: P-Tyr

FIG. 4. Calcium induction of EGFR tyrosine phosphorylation and downstream signaling. PC12 cells were treated with 50 mM KCl or 1 ng of EGF per ml for the indicated times. Lysates were immunoprecipitated with anti-EGFR pAb, and immunoprecipitated proteins (IP) (*Top*) or samples of lysates (*Bottom*) were analyzed by immunoblotting with anti-Tyr(p) antibodies. Positions of migration of prestained molecular size markers (kDa) are shown. Arrows indicate positions of the 42- and 44-kDa MAPK isoforms.

phosphorylated and activated form of MAPK (L.B.R., David D. Ginty, and M.E.G., unpublished observations). Thus, calcium influx in response to L-type VSCC activation leads to tyrosine phosphorylation of the EGFR to an extent that is sufficient to induce downstream signaling to MAPK. Notably, the dose of EGF used in this experiment is 10-fold higher than doses reported to generate physiological responses in neurons (47, 48), suggesting that the comparable level of EGFR tyrosine phosphorylation produced by calcium influx is likely to be a physiologically important mechanism of signal transduction in response to neuronal activity.

Finally, we addressed the generality and specificity of the calcium signaling response by examining whether KCl induced tyrosine phosphorylation of other growth factor receptors. KCl treatment led to tyrosine phosphorylation of the EGFR family member ErbB2 (Fig. 5A) as well as a 100-kDa protein that is recognized by an antibody generated against a consensus tyrosine kinase domain (unpublished data). In contrast, KCl did not induce tyrosine phosphorylation of the insulin receptor (unpublished data) or the NGF receptor Trk, which was robustly phosphorylated in response to NGF (Fig. 5B). These results suggest that calcium influx may activate the signal transduction pathways of other receptor tyrosine kinases in addition to the EGFR but that there is specificity in the signaling responses generated. Thus, stimulation of growth factor receptor signaling may be a general mechanism by which calcium influx generates long-term responses in cells.

DISCUSSION

Our results demonstrate that activation of VSCCs can lead to tyrosine phosphorylation of the EGFR and its association with the adaptor proteins Shc and Grb2 and the guanine nucleotide exchange factor Sos1. A number of studies have demonstrated that Shc, Grb2, and Sos1 act as signaling mediators in growth factor receptor activation of Ras. Taken together, these findings provide a mechanism by which calcium influx could activate the Ras/MAPK pathway and demonstrate that growth factor receptors can be functionally coupled to their downstream signaling pathways in response to ion-channel activation.

The mechanism by which calcium influx induces EGFR tyrosine phosphorylation is not yet clear. Although it is possible that L-type VSCC activation could lead to the calcium-induced release of EGF and autocrine stimulation of the receptor, we favor a model of ligand-independent EGFR activation by calcium for a number of reasons. The EGFR precursor is a transmembrane protein not known to be packaged in secretory vesicles (49), and we have found that a neutralizing antibody to EGF prevents tyrosine phosphoryla-

tion of the EGFR in response to EGF but not in response to L-type VSCC activation (unpublished data). Thus, calcium does not lead to EGFR tyrosine phosphorylation by autocrine release of EGF itself. We think a more likely mechanism could involve calcium activation of a cytoplasmic tyrosine kinase that could phosphorylate the receptor C-terminal tail (Fig. 6). This trans-phosphorylation of the EGFR by a calcium-responsive cytoplasmic tyrosine kinase may then activate the EGFR kinase so that it autophosphorylates on the same sites that are phosphorylated in response to EGF binding. Members of the Src family of cytoplasmic tyrosine kinases are good candidates for mediating calcium-induced tyrosine phosphorylation of the EGFR. Although we are unaware of evidence that Src directly binds the EGFR, Src transformation of fibroblasts leads to tyrosine phosphorylation of the EGFR and likely to activation of its kinase activity as well (50). In addition, Src is activated in response to ionomycin treatment of keratinocytes (51), and targeted gene disruption of the Src family member *fyn* suggests that the Fyn protein may play a role in calcium-dependent responses in the nervous system, such as synaptic potentiation and memory formation (52). Alternatively, calcium influx could increase EGFR tyrosine phosphorylation by activating an as yet uncharacterized tyrosine kinase or by inhibiting a protein tyrosine phosphatase. Whether or not calcium activates the EGFR kinase activity *per se*, it does activate EGFR signal transduction by inducing association of the receptor with the downstream signaling proteins Shc, Grb2, and Sos1.

Although calcium activation of growth factor receptor signaling is likely to involve other receptor tyrosine kinases, we have also found evidence for its specificity. For example, KCl treatment of PC12 cells does not induce tyrosine phosphorylation of the insulin receptor (unpublished data) or the NGF receptor Trk (Fig. 5B). In addition, the observed effects of calcium appear to be specific to certain cell types. We have not detected inducible tyrosine phosphorylation of the EGFR or Shc in response to KCl treatment of cortical neurons, where calcium influx may activate Ras through a specific CaM-binding GEF that is not detectable in PC12 cells (69). In other studies, ionomyucin treatment of A431 cells and extracellular calcium addition to keratinocytes inhibited EGFR tyrosine phosphorylation (53, 54). These inhibitory effects may involve calcium activation of serine/threonine kinases, since phosphorylation of the EGFR on serine and threonine residues can down-regulate both EGF binding and receptor tyrosine kinase activity (55). The cell specificity of calcium effects on EGFR tyrosine phosphorylation suggests that the stimulation we observe is not simply due to a conformational change in the EGFR induced by calcium ions, as has been described *in vitro*

FIG. 5. Calcium induction of ErbB2 but not Trk tyrosine phosphorylation. PC12 cells were treated with 50 mM KCl (lanes K), 100 ng of NGF per ml (lanes N), or 10 ng of EGF per ml (lanes E) for 3 min (lanes C, controls). Lysates were immunoprecipitated with anti-EGFR pAb or anti-ErbB2 (A) or anti-Trk (B) antibody, and precipitated proteins (IP) were analyzed by immunoblotting with anti-Tyr(p) antibodies. Positions of migration of prestained molecular size markers (kDa) are shown.

FIG. 6. Calcium induction of EGFR signal transduction. Membrane depolarization leads to calcium influx through VSCCs. This leads to tyrosine phosphorylation of the EGFR, possibly by activating a cytoplasmic tyrosine kinase such as Src. Trans-phosphorylation of the EGFR by the cytoplasmic tyrosine kinase may activate the receptor to autophosphorylate on the same sites that would be phosphorylated in response to EGF binding. Calcium-induced tyrosine phosphorylation of the EGFR leads to its association with the downstream adaptor proteins Shc and Grb2, which allows localization of the guanine nucleotide exchange factor Sos1 to the plasma membrane, where it can activate Ras and initiate signaling to MAPK.

in the presence of millimolar concentrations of Mg^{2+} or Mn^{2+} (56). The distinct effects of increased cytosolic calcium on EGFR tyrosine phosphorylation in different cell types may reflect the differential expression of calcium-responsive signaling intermediates or distinct modes of calcium entry into the cells, which can generate different signaling responses (5).

Stimulation of growth factor receptor signaling pathways in response to VSCC activation has general implications for how calcium signals may be transduced into biochemical responses in neurons. EGF and its receptor are expressed in a number of areas in the nervous system (48, 57), and expression of the EGFR family members ErbB2 (c-neu), ErbB3, and ErbB4 has also been detected in brain (58–60). In addition, a family of ligands that bind the ErbB proteins, the neuregulins, has recently been discovered, which can act as trophic factors in the nervous system (61). This widespread expression of EGFR family signaling machinery in the nervous system suggests that it could be used in response to activity-dependent calcium influx via the mechanism described here. For example, one long-term response to membrane depolarization-induced calcium influx is enhanced survival of certain neuronal populations, such as cerebellar granule cells (62, 63). EGF itself has been shown to be a survival factor for certain types of neurons as well, including cerebellar granule neurons (47, 64, 65). Thus, one mechanism by which neuronal activity may enhance neuronal survival may be through calcium activation of EGFR signaling pathways. Calcium stimulation of growth factor signaling pathways may be a general mechanism for activity-dependent regulation of survival and trophic responses in the nervous system (62, 66).

Note Added in Proof. While this manuscript was in press, others also observed that calcium influx leads to Shc phosphorylation in PC12 cells. The Shc phosphorylation event was shown to be mediated by Src (70) and/or a novel 112-kDa calcium-responsive tyrosine kinase, PYK2 (71).

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Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents

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Several non-physiologic agents such as radiation, oxidants and alkylating agents induce ligand-independent activation of numerous receptor tyrosine kinases (RTKs) and of protein tyrosine kinases at the inner side of the plasma membrane (e.g. Dévary et al., 1992; Sachsenmaier et al., 1994; Schieven et al., 1994; Coffer et al., 1995). Here we show additional evidence for the activation of epidermal growth factor receptor (EGFR), and we show activation of v-ErbB, ErbB2 and platelet-derived growth factor receptor. As a common principle of action the inducing agents such as UVC, UVB, UVA, hydrogen peroxide and iodoacetamide inhibit receptor tyrosine dephosphorylation in a thiol-sensitive and, with the exception of the SH-alkylating agent, reversible manner. EGFR dephosphorylation can also be modulated by these non-physiologic agents in isolated plasma membranes in the presence of Triton X-100. Further, substrate (EGFR) and phosphatase have been separated: a membrane preparation of cells that have been treated with epidermal growth factor (EGF) and whose dephosphorylating enzymes have been permanently destroyed by iodoacetamide can be mixed with a membrane preparation from untreated cells which re-establishes EGFR dephosphorylation. This dephosphorylation can be modulated in vitro by UV and thiol agents. We conclude that RTKs exhibit significant spontaneous protein kinase activity; several adverse agents target (an) essential SH-group(s) carried by (a) membrane-bound protein tyrosine phosphatase(s).
Keywords: N-acetylcysteine/oxidants/RTKs/tyrosine phosphatases/ultraviolet irradiation

Introduction

Adverse agents such as radiation, alkylating chemicals and oxidants induce major changes in mammalian cell gene expression (for references see Fornace, 1992; Herrlich et al., 1992; Herrlich and Rahmsdorf, 1994; Rahmsdorf, 1994; Schenk et al., 1994; Friedberg, 1995; van Dam et al., 1995). As a result of the altered programme of gene products, the cells transiently arrest the cell cycle, repair damage and prepare for proliferation, or go into apoptosis. The induction of gene expression requires the activation of specific transcription factors and the triggering of signal transduction cascades. An adverse agent will only exert such effects if it interacts with and changes the activity of a cellular component that feeds into one of the physiologic signalling pathways.

Treatment of mammalian cells with short wavelength UV light causes the activation of numerous transcription factors (Stein et al., 1989; Dévary et al., 1992, 1993; Sachsenmaier et al., 1994; M.Iordanov, K.Bender, T.Ade, W.Schmid, C.Sachsenmaier, K.Engel, M.Gaestel, H.J. Rahmsdorf and P.Herrlich, submitted). Steps preceding the activation of transcription factors have been well characterized and include several levels of protein kinases linking components at the plasma membrane to the nucleus (Dévary et al., 1992; Radler-Pohl et al., 1993; Sachsenmaier et al., 1994; Dérijard et al., 1994). Interestingly, a major part of the UV-induced signalling to the nucleus is thought to originate at the plasma membrane and involves Ras and other G-proteins. As the earliest event yet detected, various receptor tyrosine kinases (RTKs) are activated immediately following UV exposure (Sachsenmaier et al., 1994; Miller et al., 1994; Schieven et al., 1994; Warmuth et al., 1994). Direct evidence for UV-induced receptor autophosphorylation exists for the epidermal growth factor receptor (EGFR) (Sachsenmaier et al., 1994; Schieven et al., 1994; Warmuth et al., 1994), for the insulin receptor (Coffer et al., 1995) and for the T cell receptor-coupled tyrosine protein kinases (Schieven et al., 1994). Indirect evidence suggests that fibroblast growth factor receptor and interleukin-1 receptor are also activated (Sachsenmaier et al., 1994). Interestingly, the natural ligands for these receptors do not seem to be required for this type of activation. Nevertheless, the activated receptors are functional in that EGFR has been shown to be required for part of the UV-induced c-fos expression (Sachsenmaier et al., 1994), and the insulin receptor activated by UV causes Shc and IRS-1 phosphorylation as it occurs after insulin treatment (Coffer et al., 1995).

The present study is concerned with the primary molecular events triggered by UVC and other adverse agents. We show that UVA, UVB, UVC, various oxidants and the thiol-alkylating agent iodoacetamide cause functional EGFR and platelet-derived growth factor receptor (PDGFR) autophosphorylations. While we considered it unlikely that differently acting agents could inflict identical gains of function to several RTKs, and postulated that the agents should, as a common principle of action, cause a loss of function, we find indeed that all these stimuli inhibit the dephosphorylation of both EGFR and PDGFR. The interference with dephosphorylation is caused by a reversible SH-group oxidation or a non-reversible modification by alkylation, presumably at the active centre of one or several protein tyrosine phosphatases. In vitro reconstitution experiments indeed support the conclusion that membrane-associated protein tyrosine phosphatases are targeted.
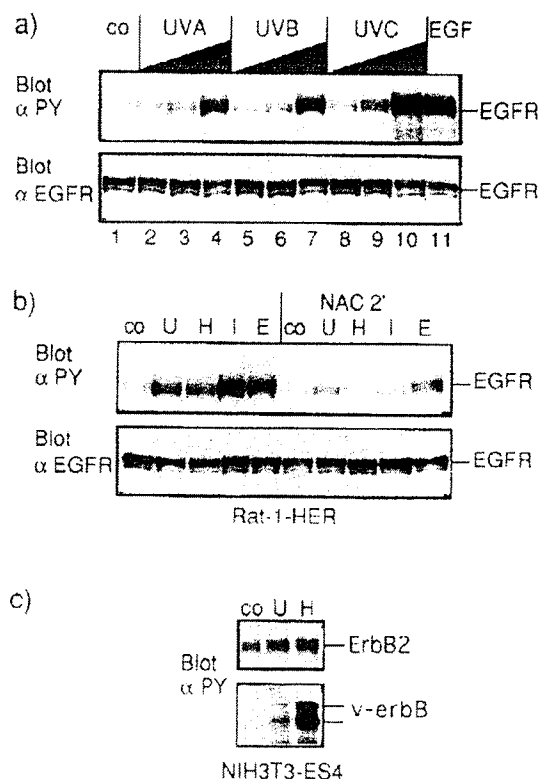
Fig. 1. Induced tyrosine phosphorylation of the EGFR by unphysiologic agents. (a) UV-irradiation induces wavelength- and dose-dependent tyrosine phosphorylation of the EGFR. Rat-1/HER cells were pretreated with 1 mM Na_3VO_4 for 1 h. They were mock-treated (co) or irradiated with 50 J/m² (lanes 2, 5 and 8), 500 J/m² (lanes 3, 6 and 9) or 5000 J/m² (lanes 4, 7 and 10) each of UVA, UVB or UVC, or treated with 2 ng/ml EGF (lane 11) for 5 min. The cells were rapidly harvested in 2× sample buffer. The extracts were analysed for tyrosine phosphorylation of the EGFR (α-PY). The membranes were stripped and reprobed with antibodies against EGFR (α-EGFR) to confirm that equal amounts of proteins were loaded. (b) N-acetylcysteine pre-treatment of cells blocks agent-induced tyrosine phosphorylation of the EGFR. After pre-treatment with 100 µM Na_3VO_4 the cells were pre-incubated with or without 30 mM N-acetylcysteine for 2 min (NAC 2'). The cells were then treated with either 1000 J/m² UVC (U), 150 µM H_2O_2 (H), 1 mM iodoacetamide (I) or 2 ng/ml EGF (E) for 5 min. Extracts were subsequently analysed as detailed in (a). (c) UV-irradiation or H_2O_2-treatment of NIH 3T3 cells, expressing the v-erbB oncogene, lead to enhanced tyrosine-phosphorylation of 66 and 72 kDa v-erbB proteins and of endogenous ErbB-2. Cells were pre-incubated with 100 µM Na_3VO_4 and then mock-treated (co), UVC-irradiated (5000 J/m²) (U) or treated with 1 mM H_2O_2 (H) for 5 min. Cells were lysed in coprecipitation lysis buffer (see Materials and methods). ErbB-2 was immunoprecipitated with a polyclonal serum ('Toby') and v-ErbB was immunoprecipitated with the polyclonal serum 1005 raised against an intracellular sequence of EGFR. The immunoprecipitated proteins were resolved by SDS–PAGE and their tyrosine phosphorylation was detected by Western blot analysis. The blot in C, lower panel, has been underexposed deliberately in order to distinguish clearly the tyrosine phosphate signals after UV and H_2O_2. As expected the receptor is phosphorylated at tyrosine residues already in untreated cells, and this phosphorylation is enhanced by UV and H_2O_2.

Results

Ligand-independent and functional autophosphorylation of the EGFR

The rapid autophosphorylation at tyrosines of the EGFR (Sachsenmaier et al., 1994 and Figure 1) has been identified as an important trigger of the activation of signalling pathways that occurs in cells following treatment with

UVC (Schorpp et al., 1984; Dévary et al., 1992; Radler-Pohl et al., 1993). Since many other adverse agents, including X-rays, UVA, UVB, mitomycin-C, alkylating agents, oxidants and antioxidants, have also been reported to induce gene transcription and to activate signal transduction pathways, we examined whether representatives of such agents could also trigger EGFR activation. Within 5 min and in a dose-dependent manner, 50–5000 J/m² of either UVA, UVB or UVC induce autophosphorylation at tyrosines of the human EGFR expressed in rat-1 cells (rat-1/HER, Figure 1a). We have not been able to reach plateau levels of phosphorylation by increasing the doses. Doses 10-fold higher resulted in further increases (not shown) and doses beyond were not feasible because of the duration of irradiations needed. The doses of UVC and UVB chosen were certainly sufficient to kill the cell. We are looking at very early events (2–5 min, while killing is a question of hours) and wish to see the bulk of EGFR phosphorylated. EGFR phosphorylation can, however, be detected with a UVC dose as low as 30 J/m² (Sachsenmaier et al., 1994; M.Iordanov, unpublished). The efficiency of UVC seems superior to that of UVA and UVB, causing levels of autophosphorylation equivalent to those induced by 2–5 ng/ml EGF. The induction was not, however, related to the killing efficiency of radiation: e.g. the colony forming ability of human fibroblasts is decreased 300 times less efficiently by UVB, and 30 000 times less efficiently by UVA, than by UVC (Vile et al., 1995). Thus the killing lesion, most likely DNA damage, is not involved in induced EGFR autophosphorylation.

Various oxidants such as hydrogen peroxide (H_2O_2) or permanganate, and the SH-alkylating agent iodoacetamide also cause rapid autophosphorylation of the EGFR (Figures 1b, 2 and 4). While UV light and oxidants induce activation within seconds (see Sachsenmaier et al., 1994; and not shown), autophosphorylation induced by iodoacetamide requires several minutes, perhaps due to slow uptake of the compound into the cell and to the slow alkylation reaction.

Activation of the EGFR by these agents appears to be functional since the Shc protein is phosphorylated (Coffer et al., 1995) and associates with the receptor (Figures 2a, 2b and 4b). Coprecipitation of Shc with the EGFR matches the degree of autophosphorylation of the receptor. While in extracts from B82L cells which lack an EGFR, Shc cannot be coprecipitated using an EGFR antibody (Figure 2a, left panel), this is possible from extracts of treated B82L cells transfected with an expression construct for the EGFR (p52 Shc isoform shown in Figure 2a, middle panel). Also in other cell lines, such as HeLa and rat-1/HER cells, autophosphorylation of EGFR after treatment with UVC or H_2O_2 is functional as it leads to binding of Shc isoforms to tyrosine-phosphorylated EGFR (Figures 2b and 4b). We have also observed UVC-induced phospholipase Cγ (PLCγ) association with the EGFR (not shown) and found that the phosphopeptide map after trypsin digestion was similar or identical after either UVC or EGF treatment (not shown). Also, the activation of downstream targets, e.g. of Ras, Erk 1 and 2, and of Elk-1 (Dévary et al., 1992; Radler-Pohl et al., 1993; Sachsenmaier et al., 1994 and see below) strongly suggests that several adverse agents lead to at least partial activation of the EGFR.

To exclude the possibility that the observed effects on EGFR autophosphorylation are due to induction of
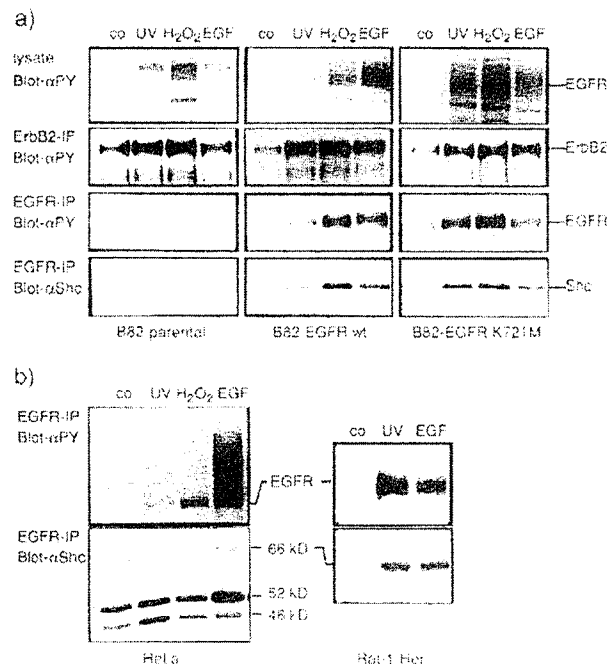
a)

b)

Fig. 2. UVC, H_2O_2 and EGF induce tyrosine phosphorylation of ErbB-2, of the EGFR and the kinase-dead EGFR mutant Lys721Met. Shc is complexed with the EGFR in treated cells. (a) B82 parental cells, or B82 cells transfected with wild type EGFR or with the kinase-dead mutant EGFR Lys721Met were pretreated with 1 mM Na_3VO_4 for 1 h and then treated with 1500 J/m² UVC, 150 μM H_2O_2 or 2 ng/ml EGF. Cells were harvested in co-immunoprecipitation buffer after 5 min. EGFR and ErbB2 were immunoprecipitated. The immuno-precipitates and aliquots of the cell extracts were analysed for tyrosine phosphorylation (upper three panels) or amount of p52 Shc protein coprecipitated with EGFR (lower panel) by Western blot analysis. (b) Cells were pre-incubated with 100 μM Na_3VO_4 for 1 h. HeLa cells were then mock-treated (co), irradiated with 1500 J/m² UVC (UV), treated with 150 μM H_2O_2 or 10 ng/ml EGF. Rat-1/HER cells were mock-treated (co), irradiated with 3000 J/m² UVC (UV) or treated with 10 ng/ml EGF. The EGFR (endogenous in HeLa, stably transfected in Rat-1/HER) was immunoprecipitated in co-immunoprecipitation buffer. The level of EGFR tyrosine-phosphorylation (upper panel) and of coprecipitated Shc (lower panel, p46, p52 and p66 Shc in HeLa, p66 Shc in Rat-1/HER) was determined by Western blot analysis.

a)

b)

Fig. 3. *N*-acetylcysteine inhibits induced phosphorylation of the PDGFR. NIH 3T3 cells overexpressing the β-PDGFR were pretreated with 100 μM Na_3VO_4 for 1 h and pre-incubated with or without 30 mM *N*-acetylcysteine for 5 min as indicated. The cells were mock-treated (–), irradiated with 5000 J/m² of UVA (A), UVB (B) or UVC (C) or treated with 1 mM H_2O_2 (H), 1 mM potassium permanganate (K) or 20 ng/ml PDGF-BB (P) for 5 min (a) or with 1 mM iodoacetamide (IAA) for 10 min (b). The cells were lysed in 2× sample buffer and protein extracts were analysed for tyrosine phosphorylation (α-PY) and amount of PDGFR (α-PDGFR) by Western blot analysis.

autocrine EGF or TGFα production, we examined the activation of v-ErbB, a mutated form of the chicken EGFR displaying a basal degree of ligand independence. When expressed in an EGFR-negative 3T3 cell line, p60 v-ErbB is efficiently tyrosine-phosphorylated after treatment of cells with UVC or H_2O_2 (Figure 1c), although it carries a truncated extracellular domain and can neither bind EGF nor other known ligands (Massoglia *et al.*, 1990). We conclude, therefore, that the adverse agents activate the EGFR in the absence of ligand and enhance the tyrosine phosphorylation state of constitutively activated p60 v-ErbB (the blot in Figures 1c lower panel has been underexposed deliberately, see legend).

Induced activation of ErbB2 and PDGFR

The results with the EGFR-negative cell line B82L prompted us to examine another member of the EGFR family: ErbB2. In B82L cells (Figure 2a) or NIH 3T3 cells (not shown), ErbB2 (the endogenous murine homologue of the neu/HER2 receptor) is phosphorylated at tyrosine residues in response to UVC or H_2O_2, but not after EGF

treatment (Figure 2a, left panel). This demonstrates, as do the results with HeLa cells (Figure 2b, left panel and Sachsenmaier *et al.*, 1994) that overexpression of tyrosine kinase receptors (as in the transfected cell lines) is not a prerequisite for UV- and oxidant-induced receptor phosphorylation. Interestingly, transfection of these cells with a catalytically inactive EGFR mutant carrying a Lys to Met exchange at position 721 (Chen *et al.*, 1987), re-establishes both UVC- and oxidant-induced autophosphorylation of the EGFR mutant receptor (as well as association with Shc) and EGF dependent activation of ErbB2 (Figure 2a, right panel). This must be due to heterodimerization of EGFR with ErbB2 (Stern and Kamps, 1988; Wada *et al.*, 1990; Qian *et al.*, 1992; Wright *et al.*, 1995), efficient phosphorylation of the EGFR by ErbB2 activated after treatment with UVC or H_2O_2, and at least some activation of the heterodimer by EGF.

To explore to what extent the induced autophosphorylation mechanism can be generalized and to examine a receptor of another class (Yarden and Ullrich, 1988), we measured the phosphorylation state of the β-PDGFR in receptor overexpressing NIH 3T3 cells following treatment with adverse agents. UVA, UVB, UVC, H_2O_2, permanganate (Figure 3a, left panel) and iodoacetamide (Figure 3b, left panel) efficiently induce tyrosine phosphorylation of the PDGFR. The magnitude resembled that achieved by 10–20 ng/ml PDGF-BB.

We conclude that activation of RTKs such as EGFR, ErbB2, PDGFR and possibly many others in response to radiation, oxidants and SH-alkylating agents may represent a general feature caused by a common underlying mechanism.

A sulfhydryl group as the common target

Ligand-independent activation of many RTKs after treatment of cells with agents as diverse as those used above suggests that the molecular event induced is common to

several receptors, that it likely effects the inactivation of a target rather than a gain of function, and that the agents share a mechanism of action in this respect. Along these lines, we first considered whether the inducing agents could share a reaction intermediate. Since UVA, UVB and the oxidants could act through reactive oxygen intermediates (UVA or UVB irradiation reduces cellular glutathione levels, Connor and Wheeler, 1987; Fuchs *et al.*, 1989; Shindo *et al.*, 1993) and their action on the transcription factor NFκB can be counteracted by antioxidant agents such as *N*-acetylcysteine (Staal *et al.*, 1990; Meyer *et al.*, 1993; Vile *et al.*, 1995), we tested several SH-group reagents for their ability to interfere with the induction of RTK autophosphorylation. The important outcome of these experiments was that all of the SH-protecting agents tested prevented the activation of either the EGFR or PDGFR. *N*-acetylcysteine (Figures 1b, 3, 4a and b), glutathione or L-cysteine (Figure 4a), as well as dithiothreitol and 2-mercaptoethanol (not shown), effectively prevented the induced autophosphorylation of either EGFR or PDGFR by UVC, H_2O_2, iodoacetamide or permanganate. Although agents like permanganate likely destroy thiol agents directly, these results nevertheless indicate that all inducing agents directly, or through a reaction intermediate, act on one or several SH-groups relevant for receptor activity. That the excess of SH-agent also interfered with the induction by EGF or PDGF is probably trivial because the function of both growth factors is thought to depend on intra- and intermolecular S-S-bridges (Johnson *et al.*, 1982, 1984; Cooke *et al.*, 1987).

The reactive SH-group could be located in the receptor molecules or in an associated protein regulating receptor activity. The slow kinetics of receptor activation by iodoacetamide could be interpreted to indicate an intracellular target. This is consistent with the outcome of the experiment with EGFR-negative NIH 3T3-2.2 cells transfected with p60 v-ErbB (see above, Figure 1c). In these cells, p60 v-ErbB is phosphorylated after UVC or oxidant treatment. Since p60 v-ErbB carries a truncated extracellular domain (Downward *et al.*, 1984; Ullrich *et al.*, 1984), the reactive SH-group in the EGFR wild type molecule must be in the v-ErbB-like portion of the EGFR molecule or in a protein interacting with this domain.

How could an SH-group in a protein be changed by adverse agents? An obvious possibility is by oxidation. Oxidation should be reversible. Indeed, the effect of UVC and H_2O_2 on the EGFR and presumably the induced modification of the relevant SH-group can be reversed by treatment of cells with high levels of *N*-acetylcysteine (30 mM) after either irradiation or H_2O_2 application (not shown; see the *in vitro* data below). As one might expect, the SH-group modification by iodoacetamide is not reversible (not shown; see also below).

Our finding that UV and oxidants induce the phosphorylation of tyrosine kinase receptors and that this phosphorylation can be inhibited by SH-agents prompted us to re-address the important question of whether the phosphorylation state of the receptor correlates with the activation state of downstream components in the signal transduction chain to the c-*fos* promoter. The findings displayed in Figure 4b and c suggest that with the cell lines used such correlation can be found, e.g. in HeLa
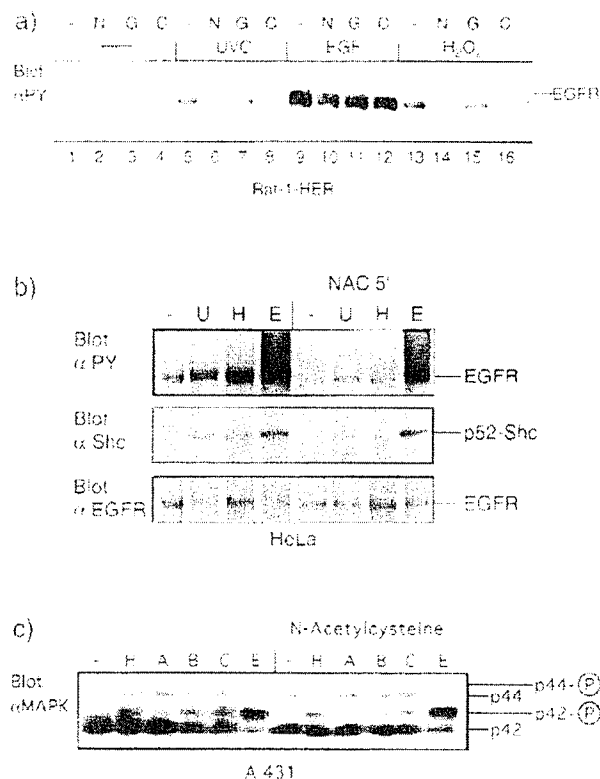
Fig. 4. SH-group-containing agents inhibit induced EGFR phosphorylation, Shc association with the receptor and Erk 1 and 2 modification. (a) Rat-1/HER cells were pretreated with 100 μM Na_3VO_4 for 1 h and incubated with 10 mM *N*-acetylcysteine (N), 10 mM reduced glutathione (G), 10 mM L-cysteine (C) or no addition (−) for 5 min. The cells were then irradiated with 1000 J/m^2 UVC, treated with 10 ng/ml EGF or with 150 μM H_2O_2. After 5 min the cells were harvested in 2× sample buffer and analysed for tyrosine phosphorylation of the EGFR by Western blotting.
(b) *N*-acetylcysteine inhibits the UV- and oxidant-induced association of p52 Shc with the EGFR. Serum starved HeLa tk$^-$ cells were pretreated with 100 μM Na_3VO_4 for 1 h and incubated with or without 30 mM *N*-acetylcysteine for 5 min. They were irradiated with 3000 J/m^2 UVC for 5 min (U) or treated with 1 mM H_2O_2 (H) or 20 ng/ml EGF (E) respectively. The EGFR was immunoprecipitated and tyrosine phosphorylation of the EGFR (upper panel), coprecipitation of Shc protein (middle panel) and the total amount of EGFR precipitated (lower panel) were analysed. (c) *N*-acetylcysteine inhibits UV- and oxidant-induced modification of Erk 1 and 2. A431 were not treated or pretreated with 30 mM *N*-acetylcysteine for 5 min. They were mock-treated (−), treated with 1 mM H_2O_2 (H) or 1000 J/m^2 UVA (A), UVB (B), or UVC (C) or treated with 10 ng/ml EGF (E) for further 5 min. Cell lysates were analysed in a Western blot with Erk 1- and 2-specific antibodies. The non-phosphorylated and phosphorylated forms of Erk 1 and 2 are indicated.

cells UV and H_2O_2 enhanced phosphorylation of the EGF receptor and association of the receptor with Shc is reduced (UV) or inhibited (H_2O_2) by pretreatment of the cells with *N*-acetylcysteine (Figure 4b) (note the loading control of total EGFR detected by Western blotting. Normalization for EGFR, particularly for the narrow first lane, shows the effects of UVC on tyrosine phosphorylation and Shc binding.) In A431 cells, *N*-acetylcysteine reduces UVC and H_2O_2 induced Erk 1 and 2 activation (Figure 4c). *N*-acetylcysteine also decreases, in NIH 3T3 cells, UV-induced c-*fos* transcription (not shown). These correlative findings support but do not unequivocally prove that the activation state of downstream components of the signal chains involved depend on the phosphoryl-

0 0,1 1 10 100 1000 µM Na₃VO₄

mock ... EGFR

UVC ... EGFR

H₂O₂ ... EGFR

EGF ... EGFR

Rat-1-HER Blot: αPY

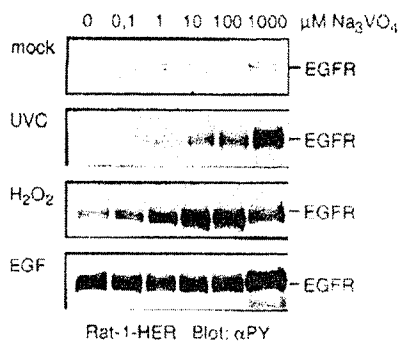**Fig. 5.** Dose-dependent increase of induced tyrosine phosphorylation of the EGFR by pretreatment with Na₃VO₄. Rat-1/HER cells were pre-incubated for 1 h with doses of Na₃VO₄ from 0 to 1000 µM. Where indicated the cells were irradiated with 3000 J/m² UVC or treated with 1 mM H₂O₂ or 2 ng/ml EGF. After 5 min tyrosine phosphorylation of the EGFR was analysed.

ation state of the receptor. In a multiple step cascade of signalling between surface receptors and nucleus, more than one step may be influenced by the adverse agents and not necessarily in the same sense. Further, the overall outcome may depend on the cell type-specific complement of enzymes controlling the cellular redox state (see Discussion).

Involvement of protein tyrosine phosphatases

Following the idea that the adverse agents should cause a loss rather than a gain of function, we considered possibilities for loss of function that could lead to increased RTK activity. A given level of receptor activity could be the result of an equilibrium between the enzymatic tyrosine kinase action and the action of a 'counteracting principle'. The adverse agents could disturb this equilibrium, shifting it favorably towards tyrosine kinase activity. A plausible 'counteracting principle' could be a protein tyrosine phosphatase. If the activity of a phosphatase or the interaction between kinase and phosphatase were the target of the adverse agents, a phosphatase inhibitor should act synergistically. We included vanadate [a non unspecific protein tyrosine phosphatase inhibitor (Swarup *et al.*, 1982)] in most assays (see legends of figures) because we then obtained somewhat better tyrosine phosphorylation signals after treatment with EGF or UV. In a systematic dose study from 0–1 mM vanadate, we found synergy with UVC or H₂O₂ in inducing EGFR (Figure 5) or PDGFR (not shown) autophosphorylation. Vanadate on its own did not cause any significant EGFR phosphorylation in experiments with rat-1/HER cells, with the exception of the highest concentration of vanadate (1 mM) and only after treating cells with this concentration for 60 min (Figure 5). UVC in the absence of vanadate caused slight increases of EGFR phosphorylation. Even concentrations as low as 0.1 µM vanadate enhanced the effects of UVC or H₂O₂ on the EGFR, much more so than it enhanced the effect of EGF (Figure 5). A possible interpretation of these data could be that vanadate and the adverse agents activate the EGFR via a common mechanism. Unfortunately, we could not reach plateau levels of activation with either vanadate or UVC, so were unable to ask whether synergy would then still occur. (It would not if the mechanism of action were identical.)

Addition of H₂O₂ to a vanadate solution leads to the

formation of pervanadate which is a more efficient inhibitor of tyrosine phosphatases than vanadate (Heffetz *et al.*, 1990). However, UV irradiation of a vanadate solution does not produce pervanadate in our hands (which can be detected as an increase of absorption between 360 and 400 nm, not shown). Nor can the UVC effect on cells be mimicked by irradiation of vanadate-containing culture medium and transfer to non-irradiated cells (not shown).

Reduced rate of dephosphorylation

To examine the involvement of protein tyrosine phosphatases in the observed effects, we measured the dephosphorylation of RTKs. To this end, cells were treated with EGF to cause autophosphorylation of the EGFR. To arrest EGFR autophosphorylation, a specific inhibitor of EGFR tyrosine kinase activity, AG1478 (Levitzki and Gazit, 1995; Böhmer *et al.*, 1995), was added and the fate of tyrosine phosphate in EGFR was monitored by Western blotting with tyrosine phosphate-specific antibodies. Dephosphorylation of the EGFR occurred with a half-time of <1 min (Figure 6a). If, however, the cells were treated with either UVA, UVB, UVC, H₂O₂ or iodoacetamide, the dephosphorylation was significantly retarded. This effect was already clearly seen at 1 min after addition of AG1478. H₂O₂ stabilized EGFR phosphate and no decrease was detected over 10 min while the other agents increased the half-time considerably (Figure 6a). A similar inhibition of EGFR dephosphorylation by UV and H₂O₂ was detected in HeLa cells (Figure 6b), suggesting that our finding with rat-1/HER cells was not due to overexpression of the receptor in these cells and that induced receptor phosphorylation in HeLa cells (Figures 2b and 4b, and Sachsenmaier *et al.*, 1994) was due to impaired dephosphorylation. Similar retardation of tyrosine phosphate hydrolysis has been observed for the PDGFR following treatments with the specific kinase inhibitor AG1296 (Levitzki and Gazit, 1995; Böhmer *et al.*, 1995) and with either UVA, UVB or UVC (Figure 6c). AG1478 completely inhibits UV- or H₂O₂-induced tyrosine phosphorylation of the EGFR (not shown), indicating that in the case of H₂O₂ (where it is relevant because of the experimental design) the inhibitor is not destroyed by the treatment.

If the prolonged half-time of dephosphorylation was the mechanism of receptor activation by the adverse agents examined here, the slow dephosphorylation should not occur in the presence of one of the SH-group carriers. This is indeed the case: UVA, UVB, UVC, hydrogen peroxide or iodoacetamide could not enhance the lifetime of the tyrosine phosphate if the cells had been pretreated briefly with N-acetylcysteine (Figures 7a and b). The SH-group sensitive step is thus probably identical to that activating the receptors and reducing the rate of their dephosphorylation.

Dephosphorylation of the EGFR in vitro

These experiments cannot help to demonstrate whether a phosphatase (counteracting receptor kinase) is inactivated, or whether the receptor is the target in that the agents change the accessibility for the phosphatase. In order to distinguish between an inactivation of a phosphatase and a change in the accessibility of the receptor substrate, we tried to separate receptor and phosphatase *in vitro*. Cells
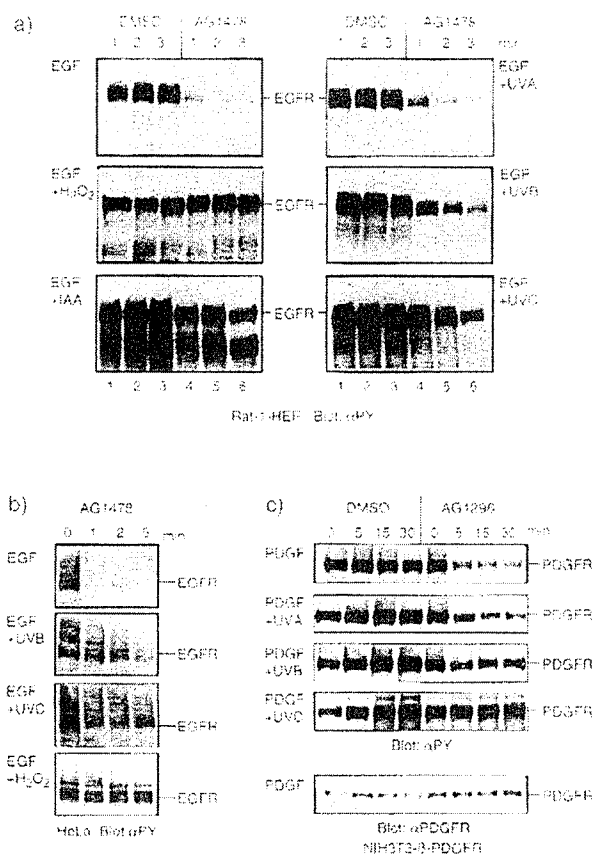
Fig. 6. (a) Treatment of rat-1/HER cells with UV. H_2O_2 or iodoacetamide inhibits the dephosphorylation of EGFR tyrosine residues. Rat-1/HER cells were pretreated with 1 mM Na_3VO_4 for 1 h. The cells were treated for 5 min with 2 ng/ml EGF or EGF in combination with 1 mM H_2O_2, 1 mM iodoacetamide (IAA) or 4000 J/m² UVA, UVB or UVC. The cells were then treated for 1 min, 2 min or 3 min (EGF, EGF + UVA, EGF + UVB, EGF + UVC) or 1 min, 3 min or 10 min (EGF + H_2O_2 and EGF + IAA) with DMSO (lanes 1, 2 and 3) or with 100 nM AG1478 (lanes 4, 5 and 6) diluted in DMSO. The cells were harvested in 2× sample buffer and protein extracts were analysed for phosphorylation of EGFR at tyrosine residues. Reduced dephosphorylation in irradiated, H_2O_2 or IAA treated cells is detectable already 1 min after addition of AG1478. Equal loading of the gels was ascertained by reprobing the blots with EGFR-specific antibodies (not shown). (b) Treatment of HeLa cells with UV or H_2O_2 inhibits EGFR dephosphorylation. HeLa tk⁻ cells were pretreated with 1 mM Na_3VO_4 for 1 h and with 20 ng/ml EGF for 5 min. They were further irradiated with 3000 J/m² UVB or UVC or treated with 1 mM H_2O_2 for 5 min. DMSO was added for 5 min (0) or AG1478 was added for 1, 2 or 5 min, as indicated. The cell lysates were analysed for phosphorylation of the EGFR at tyrosine residues. (c) Treatment of cells with UV inhibits the dephosphorylation of the PDGFR at tyrosine residues. NIH 3T3 cells overexpressing the human β-PDGFR were treated for 1 h with 10 µM Na_3VO_4. The cells were treated for 17 min with PDGF-BB (10 ng/ml) and further 3 min mock-treated or irradiated with 3000 J/m² UVA, UVB or UVC. After irradiation the PDGFR kinase activity was inhibited by addition of 50 µM AG1296 f.c. to the medium as indicated. The cells were harvested after 0, 5, 15 and 30 min, lysed in 2× sample buffer and analysed for PDGFR tyrosine phosphorylation.To check for equal loading of the gel, the membrane was reprobed with an antibody directed against the PDGFR, as demonstrated in the lowest panel for one set of samples (PDGF).

were pretreated with EGF and vanadate to obtain tyrosine-phosphorylated receptor and then disrupted by douncing in hypotonic buffer. After removal of nuclei, the membrane fraction was separated from the cytosol. EGFR was detected, as expected, in the membrane fraction. The

tyrosine phosphates of the EGFR were subject to slow excorporation whether the membranes were incubated in the absence or presence of cytosol (Figure 8a). Solubilization of the membrane fraction in a buffer containing 0.5% Triton X-100 strongly enhanced the decay of phosphate (Figure 8a). Triton X-100 apparently did not disintegrate the complex and the phosphatase activity was part of the membrane fraction as was the substrate (the phosphorylated EGFR.)

Interestingly, dephosphorylation in Triton X-100-treated membranes depended on the addition of an SH-agent. In its absence EGFR tyrosine phosphate was completely stable (Figure 8b). Either 30 mM N-acetylcysteine or 10 mM β-mercaptoethanol led to the disappearance of the phosphate from EGFR within 3 min (Figure 8b).

Although there is no proof that the *in vitro* conditions reflect the natural dephosphorylation process, experiments with radiation and oxidants suggest that they do. Treatment of the membranous fraction with UVC inhibited the dephosphorylating reaction (Figure 8c). N-acetylcysteine counteracted this inhibition (compare receptor dephosphorylation at different concentrations of N-acetylcysteine, Figure 8d and e). Vanadate synergized with UVC (Figure 8c). The inhibition of dephosphorylation by UVC could be reversed by adding an excess of N-acetylcysteine (30 mM) after the irradiation (Figure 8e). 5 mM N-acetylcysteine did not suffice (Figure 8d). The *in vitro* reactions thus mimic the cellular response in all respects.

If the assumption was correct that the membrane fractions *in vitro* contained the natural receptor–phosphatase complexes, we can conclude that the rapid dephosphorylation of the EGFR *in vivo* is not an artefact of the treatment with the kinase inhibitor. *In vitro*, kinase activity is compromised by the absence of ATP. No inhibitor was added in most experiments. Adding AG1478 did not, however, change the outcome (not shown).

The successful experiments with isolated membrane fractions suggest the existence of a membrane-bound complex of EGFR and protein tyrosine phosphatase. It is puzzling that the addition of a non-ionic detergent was needed to support effective dephosphorylation. Perhaps the order of components in their phospholipid microenvironment or the protein conformation must be changed. In order to distinguish between the alternative possibilities (UV inhibits a protein tyrosine phosphatase, or it changes the accessibility of the receptor for protein tyrosine phosphatase), we separated substrate and phosphatase. Two types of membrane preparation were obtained from A431 cells: (i) from cells that had been treated both with EGF to phosphorylate the EGFR, and with iodoacetamide to inactivate irreversibly the phosphatase. This preparation was to serve as 'substrate' and indeed showed stable EGFR tyrosine phosphate in the Western blot (Figure 9, lanes 1–3, EGFR-PY membranes); (ii) from non-treated cells to yield a membrane preparation with non-phosphorylated EGFR and active phosphatase. This membrane fraction was divided and the portions were either not treated or irradiated with UVC or UVB. Since their EGFR was not phosphorylated, the lanes are practically empty (Figure 9, lanes 13–15, phosphatase membranes). The 'substrate' preparation was then individually mixed with one of the three 'phosphatase' fractions. The mixing with non-irradiated samples led to immediate EGFR
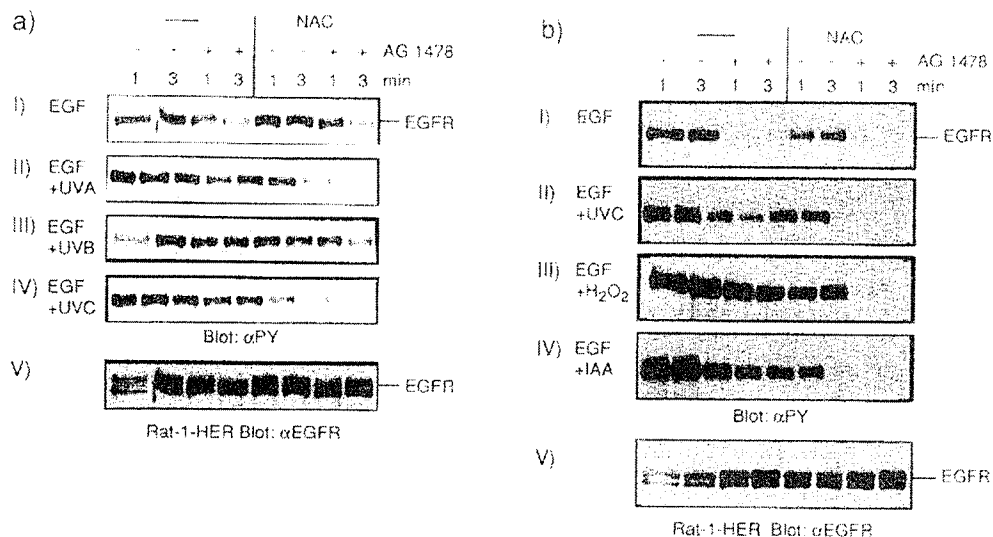
Fig. 7. Pre-treatment of cells with *N*-acetylcysteine prevents the UV-, H_2O_2- or iodoacetamide-induced inhibition of EGFR dephosphorylation.
(a) Rat-1/HER cells were pre-incubated with 1 mM Na_3VO_4 for 60 min and treated with 2 ng/ml EGF for 4 min. Then, as indicated (NAC), 30 mM *N*-acetylcysteine was added for 2 min. The cells were mock-treated or irradiated with 3000 J/m² UVA, UVB or UVC. DMSO (−) or 100 nM AG1478 (+) was added and the cells were harvested after 1 or 3 min in 2× sample buffer to be analysed for tyrosine phosphorylation of the EGFR (I–IV). Differences in tyrosine phosphorylation between samples are clearly detectable at 1 min after addition of AG1478. V shows a representative α-EGFR re-blot (panel I) after stripping. (b) Pre-treatment with Na_3VO_4 and NAC was as in (a). The cells were then treated with 2 ng/ml EGF or EGF in combination with 1 mM H_2O_2, 1 mM iodoacetamide (IAA) or 3000 J/m² UVC for 5 min. The determination of dephosphorylation kinetics was as in (a).

dephosphorylation (Figure 9, lanes 4–6), while the irradiated samples caused strongly retarded dephosphorylation (Figure 9: UVB, lanes 7–9; UVC, lanes 10–12). Thus phosphatase and EGFR substrate can be dissociated and reassembled under the conditions chosen. The phosphatase is the target of UV irradiation.

Discussion

Several laboratories including our own have shown that numerous protein tyrosine kinases are activated in response to UV irradiation (Dévary *et al.*, 1992; Sachsenmaier *et al.*, 1994; Schieven *et al.*, 1994; Miller *et al.*, 1994). Other non-physiologic agents, e.g. oxidants and alkylating agents, also strongly up-regulate tyrosine protein kinase activities (Heffetz *et al.*, 1990; Gamou and Shimizu, 1995; Brumell *et al.*, 1996; our data). Most of the affected protein kinases are RTKs and their activation by adverse agents occurs in the absence of ligands. In an attempt to explain how several diversely acting agents could induce many RTKs, we find that the equilibrium between autophosphorylation and dephosphorylation is shifted by these agents to a predominance of kinase activity by an inhibition of dephosphorylation. From the *in vitro* data it is likely that protein tyrosine phosphatases are the target of UV action. This, however, does not rule out that UV could, in addition, affect the structure of the RTKs, either changing their accessibility for phosphatases or enhancing their kinase function. In fact, it has been suggested that a protein kinase could be activated by adverse agents. As a putative mechanism the iodoacetamide- or diamide-induced oligomerization of the endoplasmic tyrosine protein kinase Ltk paralleled autophosphorylation (Bauskin *et al.*, 1991). The results with Ltk could, however, be interpreted just as well by inhibition of a phosphatase.

Our findings give rise to interesting conclusions on

common principles of action of adverse agents as well as on the regulation of signalling, and at the same time they raise several questions. Receptor tyrosine kinases can be modulated through interference with dephosphorylation. Thus the RTKs concerned must maintain spontaneous kinase activity. At least part of this spontaneous function of RTKs must be ligand-independent in that vanadate, minus or plus UVC, causes EGFR activation under starvation conditions. The truncated v-ErbB with its elevated activity is nevertheless strongly up-regulated by such treatments further documenting ligand independence. Interestingly, the laboratory of one of us (A.Ullrich) has recently detected another example of ligand-independent EGFR activation: by trimeric G-proteins (Daub *et al.*, 1996).

Receptor tyrosine kinase activation requires homo- or heterodimerization (reviewed in Schlessinger and Ullrich, 1992; Fantl *et al.*, 1993; Lemmon and Schlessinger, 1994). Furthermore, based on negative dominant mutants of EGFR, UV induction of EGFR autophosphorylation requires the receptor to dimerize, or rather, considering the kinetics of activation in seconds (Sachsenmaier *et al.*, 1994), requires that dimers pre-exist. For the same reason, RTKs and protein tyrosine phosphatases are likely to be closely associated. What then is the normal mechanism of ligand-induced RTK activation? Evidence exists for growth factors triggering self-association of the receptor subunits and several growth factors act as dimers (PDGF, see Hannink and Donoghue, 1989; Heldin *et al.*, 1989; CSF-1, see Ullrich and Schlessinger, 1990). Some receptors require their growth factors to be presented by a low affinity receptor or by a matrix component such as heparin (Spivak-Kroizman *et al.*, 1994). There may be differences between RTKs in this regard and our data indicate that a considerable fraction of the EGFR and PDGFR molecules
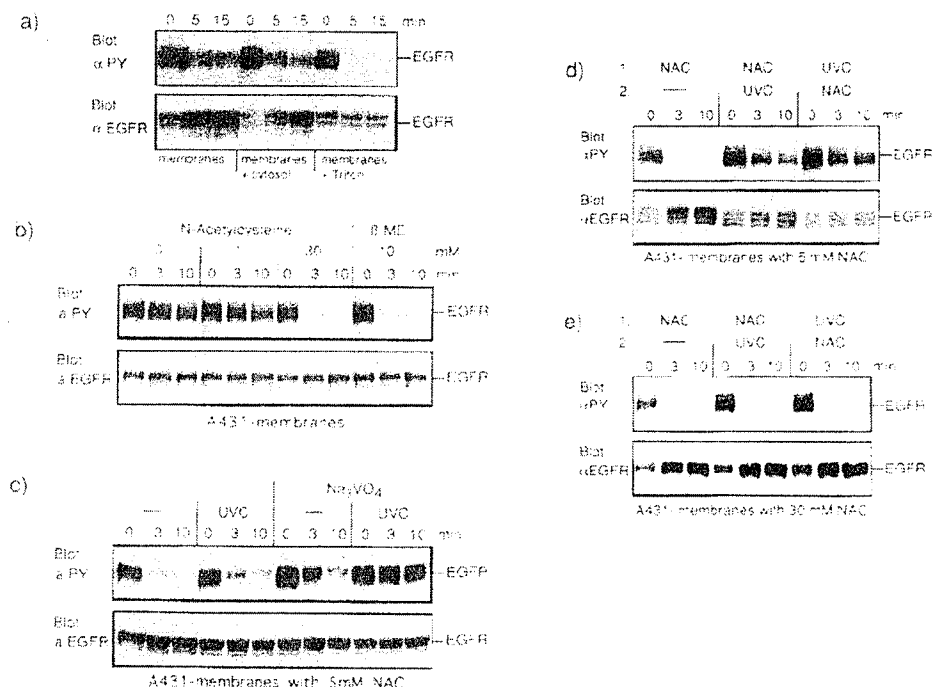
Fig. 8. EGFR dephosphorylation in isolated membranes. (a) Dependence on treatment with Triton X-100. Membranes from EGF-treated A431 cells (10 × 15 cm confluent Petri dishes) were prepared as described. Cells were lysed in 5 ml hypotonic buffer. The membranes were resuspended in a volume of 500 μl, treated with 10 mM β-mercaptoethanol and split into three 100 μl aliquots: (i) no further addition; (ii) addition of 100 μl cytosolic extract; (iii) addition of 0.5% Triton X-100 f.c. The three samples were aliquoted again and incubated at 37°C for 0, 5 or 15 min to allow dephosphorylation. The tyrosine-phosphorylation of the membrane-bound EGFR was analysed. As indicated the membrane was reprobed with an α-EGFR-specific antibody. (b) Dependence on SH-group donors. A431 membranes were prepared as in (a) and lysed in 0.5% Triton X-100. They were treated with 0, 1 or 30 mM *N*-acetylcysteine or with 10 mM β-mercaptoethanol (β-ME) for 1 min. After shifting to 37°C dephosphorylation kinetics of the EGFR were determined as in (a). (c) UV-irradiation of isolated membranes inhibits EGFR dephosphorylation. Triton X-100-lysed A431 membranes were treated with 5 mM *N*-acetylcysteine and with or without 100 mM Na₃VO₄. The membranes were then irradiated with 3000 J/m² UVC or mock-treated. After shifting to 37°C, dephosphorylation kinetics of EGFR were determined as in (a). (d and e) UV-induced inhibition of EGFR dephosphorylation is reversed by high doses of *N*-acetylcysteine after irradiation. A431 membranes were prepared as in (a) and lysed in 0.5% Triton X-100. Membranes shown in the first six lanes were treated with 5 mM *N*-acetylcysteine (d) or 30 mM *N*-acetylcysteine (e) and then not irradiated or irradiated with 3000 J/m² UVC for 3 min as indicated. Membranes shown in the last three lanes were first irradiated with 3000 J/m² UVC and then treated with 5 mM (d) or 30 mM (e) *N*-acetylcysteine. After shifting to 37°C dephosphorylation kinetics of EGFR were analysed as in (a).

are in close association in the absence of ligand and exhibit intrinsic tyrosine kinase activity.

Our experiments support heterodimerization within the ErbB family as has been reported previously (Stern and Kamps, 1988; Wada *et al.*, 1990; Qian *et al.*, 1992). Clearly, EGFR and ErbB2 exist as heterodimers, addressed by the adverse agents. Interestingly, we and others (Wright *et al.*, 1995) found enhanced EGFR tyrosine phosphorylation of EGF-treated B82 cells transfected with a kinase-deficient EGFR (Lys721Met), which argues for EGF-induced hetero-oligomerization and/or activation of the dead EGFR with/by ErbB2. The activation leads to Shc association with EGFR. This is consistent with the recent finding that intracellular retention of ErbB2 by co-expression of a single-chain antibody not only prevents neuregulin-induced signalling but also that by EGF (Graus-Porta *et al.*, 1995). ErbB2 has been reported to heterodimerize also with ErbB3 and ErbB4 suggesting a central role for ErbB2 within the family (Beerli *et al.*, 1995; Gamett *et al.*, 1995; Wallasch *et al.*, 1995). Given this role of ErbB2, we expect ErbB3 and ErbB4 also to be activated by UV and other adverse agents.

With the simultaneous activation of so many RTKs one may wonder how signalling through numerous pathways can lead to a reasonable cellular response rather than to

chaos. Indeed, UV irradiated cells mount an elaborate response of survival functions and of cell cycle arrest followed by proliferation, or, depending on the levels of various regulators, an apoptotic programme. When examining signalling pathways, we usually think of them as linear sequences of components. In reality, the intracellular communication network is likely vastly interconnected and signals from the massive stimulation of pathways by irradiation or oxidants, or else will need to pass through the network filter which may assure that only 'doable' responses are permitted.

Although we have no direct proof of whether the adverse agents used here disturb dephosphorylation of RTKs by turning the substrate into a non-accessible form or by inhibiting the protein tyrosine phosphatases, we consider the latter possibility more likely. The EGFR tyrosine phosphate substrate can be separated *in vitro* from its protein tyrosine phosphatase and the association reconstituted by mixing membrane fractions in Triton X-100. The protein tyrosine phosphatase containing fraction can be treated with UVC and mimics the behavior of the intact dephosphorylating complex *in vivo*. Further, the structure of the catalytic center of known protein tyrosine phosphatases offers a putative molecular target. A conserved cysteine in the catalytic center, e.g. of PTP1B,
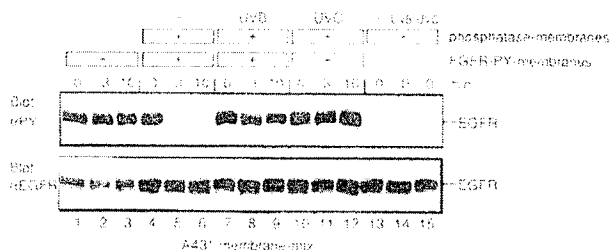
Fig. 9. UV irradiation of A431 membranes inhibits the activity of membrane-bound protein tyrosine phosphatases. Two batches of A431 membranes were prepared as follows. A substrate-membrane batch (EGFR-PY membranes) was prepared from five 15 cm Petri dishes of cells, which were pretreated with 1 mM Na_3VO_4 for 1 h, with 15 mM iodoaceteamide for 30 min and with 40 ng/ml EGF for 5 min. A second membrane batch (phosphatase membranes) was prepared from five untreated Petri dishes. Both batches were treated with 5 mM N-acetylcysteine. The phosphatase batch was treated with 10 µM Na_3VO_4. 300 µl aliquots of the EGFR-PY membranes were mixed with 300 µl hypotonic buffer containing 5 mM N-acetylcysteine (lanes 1–3), mock-treated (lanes 4–6), UVB-irradiated (3000 J/m^2) (lanes 7–9) or UVC-irradiated (3000 J/m^2) phosphatase membranes (lanes 10–12). To allow dephosphorylation, aliquots of 200 µl were incubated at 37°C for 0, 3 or 10 min. Lanes 13–15 show aliquots of the phosphatase membranes. They confirm that the EGFR in these membranes is not tyrosine-phosphorylated. The membranes were analysed for EGFR-tyrosine dephosphorylation as described in (Figure 8a). As indicated, the blot was reprobed with an α-EGFR-specific antibody.

forms a thiophosphate intermediate prior to removing the phosphate from tyrosine (Guan and Dixon, 1991). The oxidation of this cysteine or alkylation of the SH-group would inactivate the enzyme. UV irradiation of different wavelengths (including UVC) does indeed lead to oxygen intermediates, which, in a model reaction with SH-group dependent alcohol dehydrogenase, causes severe inhibition of enzyme activity accompanied by loss of 5,5′-dithio-bis-2-nitrobenzoic acid-accessible SH-groups (not shown). An action through depletion of intracellular glutathione (by high doses of UVA or UVB; Connor and Wheeler, 1987; Fuchs *et al.*, 1989; Shindo *et al.*, 1993) would have the same effect. Our data showing that SH-group agents in physiological doses (5 mM) counteract UV and oxidant induction of RTK activation and that dephosphorylation *in vitro* requires an excess of SH-groups, lends support to this interpretation. Protein tyrosine phosphatase activity has been shown to be inhibitable by H_2O_2 (Heffetz *et al.*, 1990; Sullivan *et al.*, 1994). Thus regulation of phosphatase activity via a particularly sensitive SH-group through oxidation/reduction may be an important regulatory principle of signal transduction and the protein tyrosine phosphatases may represent the sensors for a whole class of adverse agents in the micro-environment of cells.

Oxidation/reduction of critical SH-groups, often cysteine, in proteins may be more widely exploited as a regulatory principle than is currently assumed. It may regulate ion channels (Ruppersberg *et al.*, 1991) or even transcription factors (Toledano and Leonard, 1991; Xanthoudakis *et al.*, 1992). A plausible question is: what happens to the many cysteines in the extracellular domains of the RTKs (see review by Yarden and Ullrich, 1988) under oxidant/antioxidant conditions? As stated earlier, accessibility of substrate RTKs for protein tyrosine phosphatases may be an additional level of regulation, and

protein conformation of RTKs is likely determined by the state of their cysteines. Interestingly, EGF induced EGFR autophosphorylation is partly inhibited by N-acetylcysteine. We suggest that this is caused by reduction of relevant S-S-bridges. Alternatively N-acetylcysteine may enhance tyrosine phosphatase activity.

Is an RTK activated by the inhibition of dephosphorylation indistinguishably as functional as that activated by the cognate ligand? If the adverse agents simply shifted the equilibrium to protein kinase activity, functionality should be identical. The phosphorylation dependent binding of Shc and other adaptor/signalling proteins (not shown) to EGFR (Figures 2 and 4) and INSR (Coffer *et al.*, 1995) suggests normal functionality. The induction by UVC of downstream signalling with the activations of Ras, Raf, Erk 1 and 2, JNKs, and of several transcription factors (Dévary *et al.*, 1992; Radler-Pohl *et al.*, 1993; Dérijard *et al.*, 1994; Sachsenmaier *et al.*, 1994) and the dependence of UVC-induced Erk 1 and 2 activation and c-*fos* transcription on the EGFR (Sachsenmaier *et al.*, 1994; M.Iordanov *et al.*, in preparation) speak for normal functionality of such activated RTKs. On the other hand, the levels of EGFR tyrosine phosphorylation obtained by EGF, UVA, UVB and UVC do not correlate well with the efficiency of gene induction by these agents. For instance, UVA and UVB cause almost as efficient an RTK phosphorylation as UVC, but only poorly induce c-*fos* expression (activation of NFκB-dependent transcription and of c-*fos* transcription by UVB requires doses raised by one to two orders of magnitude as compared with UVC; Stein *et al.*, 1989; Shah *et al.*, 1993; Vile *et al.*, 1995; A.Knebel, unpublished). Also, EGF activates genes better than UVC. While for UVC it is clear that dose-dependent DNA damage within the transcribed gene counteracts gene expression, no such explanation can be given for UVA in the dose range used. We consider two hypotheses as plausible: adverse agents in contrast to a specific growth factor induce a multitude of RTKs and downstream signalling pathways. It is likely that the pathways cross-talk intensively and thus modify the outcome. As a result, the stimulation by EGF may activate strong signalling to the nucleus, while part of the signalling induced by e.g. UVC may be neutralized and not reach the nucleus. Alternatively, it is possible that RTKs, as well as cytoplasmic tyrosine kinases, are under dual control by different protein tyrosine phosphatases, inhibitory and stimulatory. Adverse agents, in contrast to the growth factor could affect, perhaps differentially, both types of tyrosine phosphatase. Indeed, several experiments with inhibitors, negative-dominant versions of tyrosine phosphatases and with specific transmembrane protein tyrosine phosphatases support the existence of protein tyrosine phosphatases necessary and stimulatory for signal transduction. For instance, Src is activated by dephosphorylation of Tyr527 (Zheng *et al.*, 1992). Vanadate inhibits IFNα-induced activation of transcription factors (David *et al.*, 1993), but stimulates IFNγ-induced activation (Igarashi *et al.*, 1993). Negative-dominant protein tyrosine phosphatase (PTP1D, SH-PTP2) blocks insulin and EGF signalling (Milarski and Saltiel, 1994; Noguchi *et al.*, 1994; Bennet *et al.*, 1996) and protein tyrosine phosphatase can act as an adaptor between PDGFR and Grb2 (Li *et al.*, 1994). The *Drosophila* protein tyrosine phosphatase

homologue corkscrew is an obligatory intermediate in the action of the PDGFR homologue torso (Perkins et al., 1992; Perrimon, 1993). CD45, a ligand-dependent protein tyrosine phosphatase required for lymphocyte signalling, removes an inhibitory phosphate from the tyrosine kinase Lck (Hurley et al., 1993; Desai et al., 1994). The patterns of EGFR and INSR phosphorylation after treatment of cells with H_2O_2 and/or vanadate have been compared and have been found to be identical with those induced by the natural ligands, but differences may have escaped detection (Heffetz et al., 1992; Gamou and Shimizu, 1995). We hypothesize currently that different protein tyrosine phosphatases are differentially sensitive to reaction intermediates produced by adverse agents, and thus the signal transduction result may vary. What applies to the membrane-associated protein tyrosine phosphatases could well be true for cytoplasmic SH-sensitive phosphatases in the signalling chain as well. This hypothesis may also explain why not only oxidants but also antioxidants can, in certain cells, cause activation of transcription factors and transcription of genes. While steps following directly the phosphorylation of EGFR (e.g. association with Shc) respond to UVC and N-acetylcysteine as does the EGFR itself, the effect of antioxidants or oxidants on steps further downstream is less predictable. The more steps that are interspersed between primary reaction at the cell surface and endpoint measured, the more possibilities of SH-sensitive and perhaps opposing interferences exist. Nevertheless, in 3T3 cells and in A431 cells, Erk activity and c-fos transcription behaved accordingly. But this cannot be generalized, perhaps due to differences in the complement of oxido-reduction sensitive components in different cells. In fact, antioxidants have been reported to reduce NFκB activation but to enhance c-fos and c-jun transcription (Meyer et al., 1993; Schenk et al., 1994). An interesting case has been reported concerning the action of PDGF on vascular smooth muscle cells. As in our observations with adverse agents, extracellular and intracellular antioxidants block induction by the natural growth factor of several downstream events (Sundaresan et al., 1995). Growth factors (here PDGF) seem to generate transiently increased levels of H_2O_2 with a peak at five min. It is not clear how H_2O_2 is generated. But certainly the elevated concentrations should be highly localized to ensure growth factor specificity. This observation puts the action of adverse agents into an even more persuasive physiological context.

Material and methods

Cell culture and treatment of cells

The following transfected and non-transfected cell lines were used: HeLa tk⁻ cells (Angel et al., 1987); non-transfected B82 mouse L cells and B82 cells expressing the human wild-type EGFR or a mutant version of the receptor (EGFR K 721 M) (Chen et al., 1987); rat-1/HER cells (rat-1 fibroblasts expressing the human EGFR, Wasilenko et al., 1991); NIH 3T3-ES4 cells, expressing the v-erbB oncogene (Massoglia et al., 1990); NIH 3T3-β-PDGFR cells; and A431 cells, a human epidermal carcinoma cell line expressing large amounts of the human EGFR (Ullrich et al., 1984). All cells were grown at 37°C and 6% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). Four to six hours before an experiment the medium was exchanged for DMEM without phenol red and without FCS. Where indicated, the cells were pre-incubated with freshly dissolved Na_3VO_4 pH 10 at various concentra-

tions for 60 min. The cells were mock-treated or irradiated through the medium, or antioxidants, oxidants or growth factors were added. UV irradiation was performed with lamps from Vetter, Wiesloch, with the following characteristics: UVA (UVL 15), half-maximal λ 335–370 nm, 11.5 W/m² at a distance of 8 cm; UVB (UVM 15), half-maximal λ 285–330 nm, 9 W/m² at a distance of 8 cm; UVC (UVK 15), half-maximal λ 253–255 nm, 10 W/m² at a distance of 8 cm.

Chemicals and antibodies

The α-EGFR antibody 528 and protein-G⁻–agarose were from Dianova, Hamburg; the α-EGFR antibody 1005 (used for immunoprecipitation of v-ErbB) and the polyclonal α-Erk 1 and 2 antibody were from Santa Cruz, CA; PY20, polyclonal α-Shc antibody, monoclonal α-EGFR and monoclonal α-PDGFR were from Transduction Laboratories, Exeter, UK; horseradish peroxidase-linked antisera against rabbit or mouse immunoglobulins were from Dako, Hamburg; and polyclonal antiserum against ErbB2/HER2 ('Toby') was kindly provided by Nancy Hynes, Basel. The receptor kinase inhibitors AG1296 and AG1478 were kindly provided by Alexander Levitzki, Jerusalem. Protease inhibitors, human recombinant EGF and Na_3VO_4 were from Sigma, Munich; PDGF-BB was from Biomol, Hamburg; H_2O_2 and potassium-permanganate were from Merck, Darmstadt; and ECL films and ECL detection reagent were from Amersham, Braunschweig.

Western blot analyses

5 cm dishes of confluent cells were treated and the medium was removed. The cells were immediatly lysed in warm 2× sample buffer (1× sample buffer is: 10% glycerol, 2% SDS, 0.08 M Tris pH 6.8, 2% β-mercaptoethanol, 0.025% bromphenol blue). The lysate was sonicated to shear the DNA and aliquots were boiled for 5 min. The proteins were resolved by SDS–PAGE (7.5% acrylamide, except for Erk 1 and 2 shift analysis where 10% acrylamide was used). After transferring the proteins onto Immobilon-PDVF membrane (Millipore, Bedford, UK) immunostaining was performed, using the antibody dilutions recommended by the manufacturer. Counterstaining was performed with horseradish peroxidase-linked antibodies, which were visualized using ECL detection reagent. In all cases, unblotted parts of the SDS-gels were Coomassie stained to confirm that equal amounts of proteins were loaded.

Co-immunoprecipitation

After treatment, confluent 15 cm dishes of cells were washed with PBS and lysed in 1 ml ice-cold co-immunoprecipitation buffer: 30 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% Na-deoxycholate, 10 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM freshly made Na_3VO_4 pH 10. Nuclei were pelleted and 40 μl aliquots were collected from the supernatant for Western blot analyses. From the remaining supernatant the receptors were immunoprecipitated for 1 h. The protein-G⁺–agarose pellets were washed three times with co-immunoprecipitation buffer and finally once with wash-buffer (25 mM Tris pH 7.4, 1 mM EDTA). The pellets were boiled in 40 μl 1× sample buffer for 5 min, and the proteins were resolved by SDS-PAGE, followed by Western blot analysis.

Preparation of A431 membranes and dephosphorylation of EGFR in vitro

The preparation is a modification of a method described (Bar-Sagi et al., 1988). 10 confluent dishes of A431 cells were pretreated with 1 mM Na_3VO_4 for 1 h to avoid dephosphorylation during membrane preparation. The cells were treated with 20 ng/ml EGF for 10 min (or not treated). All subsequent steps were performed on ice. The cells were washed twice with ice-cold Ca^{2+}-and Mg^{2+}-free PBS (PBS^{2-}: 137 mM NaCl, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4) and collected in a total volume of 50 ml PBS⁻. The cells were pelleted, resuspended and incubated in hypotonic buffer (20 mM Tris pH 7.5, 10 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF) for 30 min. The cells were dounced with 70–100 strokes and the nuclei were pelleted (3000 g for 5 min). The supernatant was centrifuged at 100 000 g for 25 min. The membranous sediment was resolved in hypotonic buffer with or without 0.5% Triton X-100 using a syringe with a 0.6×26 mm needle. The membranes were aliquoted and treated with antioxidants, oxidants, Na_3VO_4, UV or H_2O_2. In the dephosphorylation assay the membranes were shifted to 37°C and vigorously agitated at this temperature on an Eppendorf shaker. The dephosphorylation was stopped by addition of an equal volume of hot 2× sample buffer. The membrane extracts were analysed by Western blot analysis for the amount and tyrosine phosphorylation of the EGFR.

Acknowledgements

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Gβγ Subunits Mediate Src-dependent Phosphorylation of the Epidermal Growth Factor Receptor

A SCAFFOLD FOR G PROTEIN-COUPLED RECEPTOR-MEDIATED Ras ACTIVATION*

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In many cells, stimulation of mitogen-activated protein kinases by both receptor tyrosine kinases and receptors that couple to pertussis toxin-sensitive heterotrimeric G proteins proceed via convergent signaling pathways. Both signals are sensitive to inhibitors of tyrosine protein kinases and require Ras activation via phosphotyrosine-dependent recruitment of Ras guanine nucleotide exchange factors. Receptor tyrosine kinase stimulation mediates ligand-induced receptor autophosphorylation, which creates the initial binding sites for SH2 domain-containing docking proteins. However, the mechanism whereby G protein-coupled receptors mediate the phosphotyrosine-dependent assembly of a mitogenic signaling complex is poorly understood. We have studied the role of Src family nonreceptor tyrosine kinases in G protein-coupled receptor-mediated tyrosine phosphorylation in a transiently transfected COS-7 cell system. Stimulation of Gi-coupled lysophosphatidic acid and α2A adrenergic receptors or overexpression of Gβ1γ2 subunits leads to tyrosine phosphorylation of the Shc adapter protein, which then associates with tyrosine phosphoproteins of approximately 130 and 180 kDa, as well as Grb2. The 180-kDa Shc-associated tyrosine phosphoprotein band contains both epidermal growth factor (EGF) receptor and p185^{neu}. 3–5-fold increases in EGF receptor but not p185^{neu} tyrosine phosphorylation occur following Gi-coupled receptor stimulation. Inhibition of endogenous Src family kinase activity by cellular expression of a dominant negative kinase-inactive mutant of c-Src inhibits Gβ1γ2 subunit-mediated and Gi-coupled receptor-mediated phosphorylation of both EGF receptor and Shc. Expression of Csk, which inactivates Src family kinases by phosphorylating the regulatory carboxyl-terminal tyrosine residue, has the same effect. The Gi-coupled receptor-mediated increase in EGF receptor phosphorylation does not reflect increased EGF receptor autophosphorylation, assayed using an autophosphorylation-specific EGF receptor monoclonal antibody. Lysophosphatidic acid stimulates binding of EGF receptor to a GST fusion protein containing the c-Src SH2 domain, and this too is blocked by Csk expression. These data suggest that Gβγ subunit-mediated activation of Src family nonreceptor tyrosine kinases can account for the Gi-coupled receptor-mediated tyrosine phosphorylation events that direct recruitment of the Shc and Grb2 adapter proteins to the membrane.

The low molecular weight G protein Ras functions as a signaling intermediate in many pathways involved in the regulation of cellular mitogenesis and differentiation. Ras activation by growth factor receptors that possess intrinsic tyrosine kinase activity follows ligand-induced phosphorylation of specific docking sites on the receptor itself or adapter proteins, such as Shc and insulin receptor substrate-1, which serve to recruit Ras guanine nucleotide exchange factors to the plasma membrane (1, 2). Recently, several receptors that couple to heterotrimeric G proteins, including the lysophosphatidic acid (LPA)[1] (3, 4), α-thrombin (5), angiotensin II (6, 7), α2A adrenergic (AR) (8, 9), M2 muscarinic acetylcholine, D2 dopamine, and A1 adenosine receptors (10), have been shown to mediate Ras-dependent mitogenic signals. In COS-7 cells, Ras-dependent activation of mitogen-activated protein kinases via the α2A AR, M2 muscarinic acetylcholine, D2 dopamine, and A1 adenosine receptors is mediated largely by Gβγ subunits released from pertussis toxin-sensitive G proteins (8, 9). These Gβγ subunit-mediated signals are sensitive to inhibitors of tyrosine protein kinases (8), associated with increased tyrosine protein phosphorylation, and dependent upon recruitment of Ras guanine nucleotide exchange factors to the membrane (9), indicating that the pathway converges with the receptor tyrosine kinase pathway at an early point.

G protein-coupled receptors have been shown to mediate rapid tyrosine phosphorylation of several proteins that participate in mitogenic signal transduction. The thyrotropin-releasing hormone (11), endothelin 1 (12), LPA, and α2A AR receptors (9) stimulate tyrosine phosphorylation of the Shc adapter protein. This effect can be mimicked by the transient overexpression of Gβγ subunits (9, 13) and correlates with Shc-Grb2 complex formation (9, 12) and the recruitment of Ras guanine nucleotide exchange factor activity (9). In addition, recent re-

[1] The abbreviations used are: LPA, lysophosphatidic acid; AR, adrenergic receptor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; PI3K, phosphatidylinositol 3-kinase.

Gβγ Subunit-mediated Tyrosine Phosphorylation of EGF Receptor

ports have described G protein-coupled receptor-mediated tyrosine phosphorylation of insulin receptor substrate-1 (14), focal adhesion kinase (4, 15), and several receptor tyrosine kinases, including the platelet-derived growth factor (PDGF) receptor (16), EGF receptor, p185^{neu} (17), and insulin-like growth factor-1 receptor (14).

The mechanism whereby G protein-coupled receptors stimulate tyrosine protein phosphorylation is poorly understood. The observation that the receptors for PDGF (16) and EGF (17) undergo tyrosine phosphorylation following G protein-coupled receptor activation has led to the hypothesis that the intrinsic tyrosine kinase activity of these receptors becomes activated by an unknown mechanism. G protein-coupled receptor-mediated activation of nonreceptor tyrosine kinases has also been reported. Recently, activation of Src family kinases by the α-thrombin (18), LPA (19), angiotensin II (20), N-formyl methionyl peptide chemoattractant (21), α2A AR (18, 19), and M1 muscarinic acetylcholine (18) receptors has been reported. Furthermore, inhibition of Src family kinases has been shown to inhibit angiotensin II-stimulated Ras (22) and phospholipase C-γ1 (23) activation in rat aortic smooth muscle cells, LPA and α2A AR-stimulated MAP kinase activation in COS-7 cells (19), M1 and M2 muscarinic acetylcholine-stimulated MAP kinase activation in avian B cells (24), and endothelin-1-stimulated transcriptional activation in rat glomerular mesangial cells (25).

We have previously shown in transiently transfected COS-7 cells that pertussis toxin-sensitive G protein-coupled receptors mediate Gβγ subunit-dependent activation of c-Src and that inhibition of Src family kinases by cellular expression of Csk antagonizes G protein-coupled receptor-mediated MAP kinase activation (19). Here, we examine the role of Src family nonreceptor tyrosine kinases in mediating Gβγ subunit-dependent tyrosine phosphorylation of receptor tyrosine kinases and Shc. Our data suggest that activation of Src family kinases by G protein-coupled receptors can account for the G_i-coupled receptor-mediated tyrosine phosphorylation events that direct recruitment of the Shc and Grb2 adapter proteins to the membrane using the EGF receptor as a scaffold.

EXPERIMENTAL PROCEDURES

DNA Constructs—The cDNA encoding the α2A AR was cloned in our laboratory. The cDNAs encoding Gβ1 and Gγ2 were provided by M. Simon (California Institute of Technology, Pasadena, CA). The cDNA encoding human p60$^{c\text{-}src}$ was provided by D. Fujita (University of Calgary, Alberta, Canada), and the cDNA encoding p50^{csk} was provided by H. Hanafusa (Rockefeller University, New York, NY). The constitutively activated Y530F p60$^{c\text{-}src}$ (TAC(Y) → TTC(F)), in which the regulatory carboxyl-terminal tyrosine residue has been mutated, and catalytically inactive K298M p60$^{c\text{-}src}$ (AAA(K) → ATG(M)) mutants were prepared as described (19). All cDNAs were subcloned into pRK5 or pcDNA eukaryotic expression vectors for transient transfection.

Cell Culture and Transfection—COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μg/ml gentamicin at 37 °C in a humidified 5% CO_2 atmosphere. Transfections were performed on 80–90% confluent monolayers in 100-mm dishes. For transient transfection, cells were incubated at 37 °C in 4 ml serum-free Dulbecco's modified Eagle's medium containing 6–10 μg of DNA/100-mm dish plus 6 μl of LipofectAMINE reagent (Life Technologies, Inc.)/μg of DNA. Empty pRK5 vector was added to transfections as needed to keep the total mass of DNA added per dish constant within an experiment. After 3–5 h of exposure to the transfection medium, monolayers were refed with growth medium and incubated overnight. Transfected monolayers were serum starved in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin and 10 mM Hepes, pH 7.4, for 16–20 h prior to stimulation. Assays were performed 48 h after transfection. LipofectAMINE transfection of COS-7 cells consistently resulted in transfection efficiencies of greater than 80% (data not shown). Transient expression of Gβ1 and Gγ2 subunits, Csk, and mutant c-Src proteins was confirmed by

immunoblotting of transfected whole cell lysates using commercially available antisera.

Immunoprecipitation and Immunoblotting—Stimulations were carried out at 37 °C in serum-free medium as described in the figure legends. After stimulation, monolayers were washed once with ice-cold phosphate-buffered saline and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 100 μM NaVO$_4$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, 10 μg/ml leupeptin) for immunoprecipitation under nondenaturing conditions or RIPA/SDS buffer (RIPA buffer containing 0.1% SDS) for immunoprecipitation under denaturing conditions. Cell lysates were sonicated briefly, clarified by centrifugation, and diluted to a protein concentration of 2 mg/ml. Immunoprecipitations from 1 ml of lysate were performed using the appropriate primary antibody plus 50 μl of a 50% slurry of protein G plus/protein A agarose (Oncogene Science) agitated for 1 h at 4 °C. Immune complexes were washed twice with ice-cold RIPA buffer and once with phosphate-buffered saline, denatured in Laemmli sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Shc immunoprecipitations were performed using rabbit polyclonal anti-Shc antibody (Transduction Laboratories). EGF receptor was immunoprecipitated using monoclonal anti-EGF receptor antibody (Transduction Laboratories), and p185^{neu} was immunoprecipitated using rabbit polyclonal anti-HER2 (Santa Cruz Biotechnology).

Tyrosine phosphorylation or the presence of coprecipitated proteins was detected by protein immunoblotting. Phosphotyrosine was detected using a 1:1000 dilution of horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody (Transduction Laboratories). Shc protein was detected using a 1:1000 dilution of rabbit polyclonal anti-Shc IgG (Transduction Laboratories), p185^{neu} was detected using a 1:1000 dilution of rabbit polyclonal anti-HER2 IgG (Santa Cruz Biotechnology), and Grb2 was detected using a 1:1000 dilution of rabbit polyclonal anti-Grb2 IgG (Santa Cruz Biotechnology), each with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Corp.) as secondary antibody. C-Src was detected using 1:500 dilution of mouse monoclonal anti-c-Src antibody 327 with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Laboratories) as secondary antibody. EGF receptor was detected using 1:1000 dilution of sheep anti-human EGF receptor IgG with horseradish peroxidase-conjugated donkey anti-sheep IgG (Jackson Laboratories) as secondary antibody. Immunoblots for autophosphorylated EGF receptor were performed using mouse monoclonal anti-activated EGF receptor IgG (26) with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Laboratories) as secondary antibody. Immune complexes on nitrocellulose were visualized by enzyme-linked chemiluminescence (Amersham Corp.) and quantified by scanning laser densitometry.

GST Fusion Proteins Containing the c-Src SH2 and SH3 Domains— GST fusion proteins containing the human c-Src SH2 (amino acids 144–249), SH3 (amino acids 87–143), or SH2 and SH3 (amino acids 87–249) domains were prepared as GST Sepharose conjugates as described previously (27). For the detection of c-Src SH2 or SH3 domain-binding proteins, appropriately transfected and stimulated COS-7 cells were lysed in RIPA/SDS buffer containing 5 mM dithiothreitol, sonicated, clarified by centrifugation, precleared with 6 μg/ml GST Sepharose for 1 h and incubated with 6 μg/ml of the GST fusion protein Sepharose for 3 h at 4 °C. After incubation, fusion protein complexes were washed twice with ice-cold RIPA buffer and once with phosphate-buffered saline, denatured in Laemmli sample buffer, and resolved by SDS-PAGE. Coprecipitated tyrosine phosphoproteins and EGF receptor were detected by protein immunoblotting as described.

RESULTS

G_i-coupled Receptors and Gβγ Subunits Mediate Formation of a Mitogenic Signaling Complex Containing EGF Receptor, Shc, and Grb2—As shown in Fig. 1A, stimulation of endogenous LPA receptors in COS-7 cells leads to a rapid 3–5-fold increase in tyrosine phosphorylation of each of the three Shc isoforms. The phosphorylation is maximal within 2 min of stimulation and declines slowly thereafter (9, 19). Under nondenaturing conditions, Shc coprecipitates with two major tyrosine phosphoprotein bands of approximately 130 and 180 kDa and with the adapter protein Grb2. The association of Shc with the p130 and p180 phosphoproteins is modulated with a time course that parallels the time course of Shc phosphorylation and Shc-Grb2 complex formation, suggesting that LPA stimu-
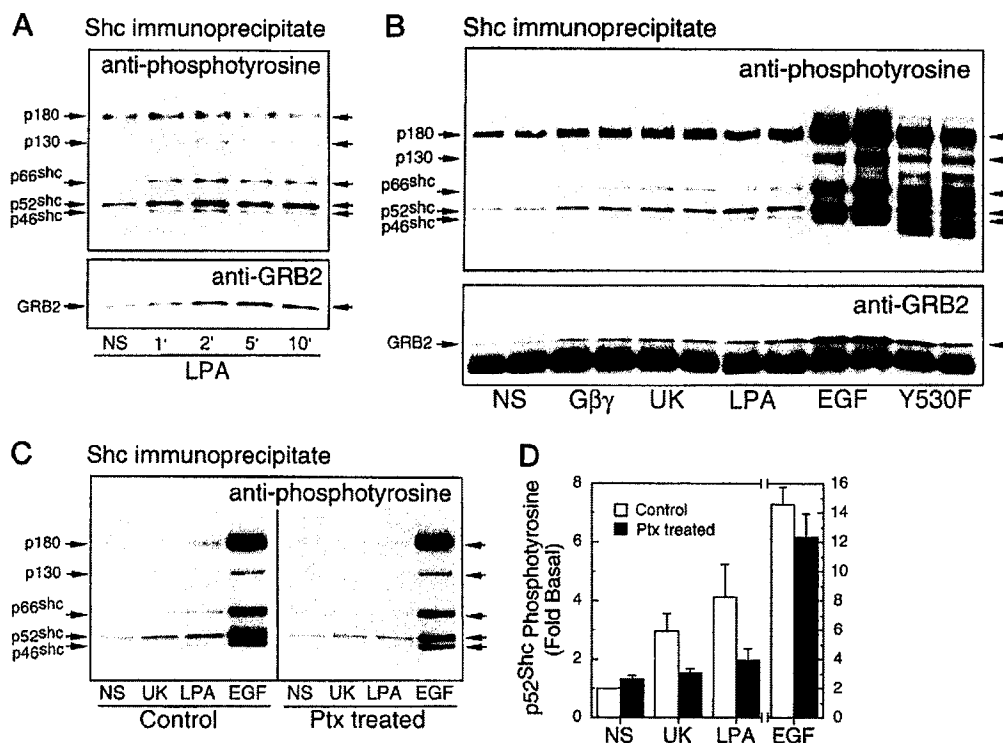
FIG. 1. **Gᵢ-coupled receptor- and Gβγ subunit-stimulated association of Shc with p130 and p180 tyrosine phosphoproteins and Grb2 in COS-7 cells.** *A*, time course of LPA-stimulated Shc tyrosine phosphorylation and association with p130 and p180 tyrosine phosphoproteins and Grb2. Serum-starved cells were stimulated for the indicated times with LPA (10 μM). Immunoprecipitates of Shc from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine (*upper panel*) or anti-Grb2 (*lower panel*) as described. The position of tyrosine phosphorylated Shc isoforms, Shc-associated p130 and p180 phosphoproteins, and Grb2 are as indicated. *B*, effect of α2A AR, LPA, or EGF receptor stimulation and Gβγ subunit or Y530F p60^c-src expression on Shc tyrosine phosphorylation and association with p130 and p180 tyrosine phosphoproteins and Grb2. Cells were transiently transfected with empty pRK5 vector, Gβ1 and Gγ2, α2A AR, or Y530F p60^c-src as described. Duplicate plates of serum-starved cells were stimulated for 2 min with the α2A AR agonist UK14304 (*UK*, 10 μM), LPA (10 μM), or EGF (10 ng/ml) as indicated. Immunoprecipitates of Shc from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine (*upper panel*) or anti-Grb2 (*lower panel*) as described. *C*, pertussis toxin-sensitivity of α2A AR- and LPA-stimulated Shc, p130, and p180 tyrosine phosphorylation. Cells were transiently transfected with empty pRK5 vector or α2A AR and serum-starved overnight in the presence or the absence of pertussis toxin (*Ptx*, 100 ng/ml) prior to stimulation with LPA, UK14304, or EGF as indicated. Immunoprecipitates of Shc from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine. *D*, quantitation of the effects of pertussis toxin treatment on α2A AR- and LPA-stimulated tyrosine phosphorylation of p52^shc. UK14304-, LPA-, and EGF-stimulated p52shc phosphorylation was determined as described. Autoradiographs were quantified by scanning laser densitometry, and the data were presented as fold increase over nonstimulated or empty pRK5 vector transfected controls. The data shown represent the means ± S.E. for three separate experiments. *NS*, nonstimulated.

lation induces association of these proteins. As shown in Fig. 1*B*, similar increases in Shc phosphorylation and Shc-p180 association result from transient expression of Gβ1γ2 subunits or stimulation of endogenous LPA or transiently expressed α2A AR receptors. Stimulation of endogenous EGF receptors or transient overexpression of a constitutively activated mutant human c-Src (Y530F p60^c-src; Refs. 28–30) has similar, although more robust effects, indicating that activation of either the receptor tyrosine kinase or nonreceptor Src kinase can mimic the G protein-mediated effects. As shown in Fig. 1 (*C* and *D*), Gᵢ-coupled receptor-mediated but not EGF receptor-mediated Shc phosphorylation and Shc-p180 association are pertussis toxin-sensitive in these cells.

Because G protein-coupled receptor-mediated tyrosine phosphorylation of PDGF receptor (16), EGF receptor, and p185^neu (17) has been reported, we performed immunoblots for EGF receptor and p185^neu on Shc immunoprecipitates from nondenatured cell lysates following stimulation of LPA, α2A AR, or EGF receptors to determine whether these receptor tyrosine kinases are present in the Shc-associated p180 phosphotyrosine band. COS-7 cells lack detectable expression of PDGF receptor (31). As shown in Fig. 2*A*, EGF receptor is not detectable in Shc immunoprecipitates from nonstimulated cells, but stimulation of either Gᵢ-coupled receptor results in Shc-EGF

receptor coprecipitation. In contrast, Shc-p185^neu complexes are present in nonstimulated cells and do not increase detectably following LPA or α2A AR receptor stimulation. As expected, EGF stimulation results in both Shc-EGF receptor and Shc-p185^neu association, which may reflect heterodimerization and transphosphorylation of the two related receptor tyrosine kinases (32). As shown in Fig. 2*B*, the tyrosine phosphorylation states of Shc, EGF receptor, and p185^neu, determined following direct immunoprecipitation of each protein, reflect the changes in Shc-receptor tyrosine kinase association. Shc and EGF receptor phosphorylation is increased following LPA, α2A AR, or EGF receptor stimulation. P185^neu exhibits significant basal tyrosine phosphorylation, consistent with the detection of Shc-p185^neu complexes in nonstimulated cells, which detectably increases only following EGF receptor stimulation.

To confirm that Shc, Grb2, and EGF receptor directly associate following Gᵢ-coupled receptor stimulation, EGF receptor immunoprecipitates were assayed for the presence of Shc and Grb2 following LPA stimulation. As shown in Fig. 2*C*, stimulation with either LPA or EGF resulted in the association of Shc and Grb2 with EGF receptor. Gᵢ-coupled receptor-induced association of Src family nonreceptor tyrosine kinases with Shc has been reported (19, 21). As shown, c-Src can also be detected in EGF receptor immunoprecipitates from LPA- or EGF-stim-
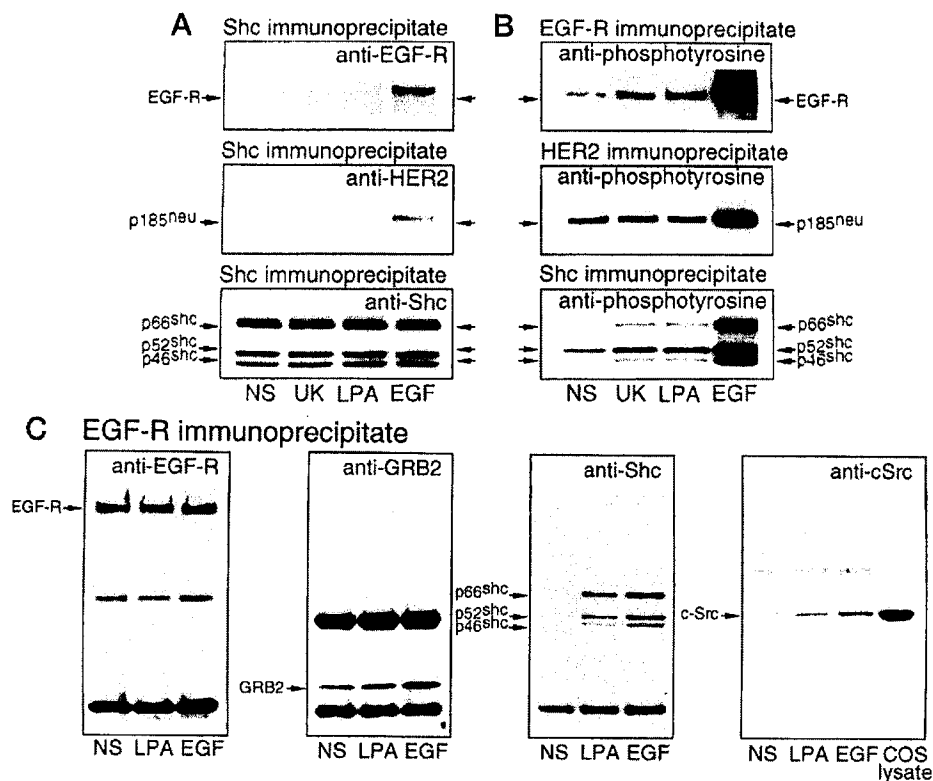
FIG. 2. Correlation between G$_i$-coupled receptor-stimulated EGF receptor and p185^{neu} tyrosine phosphorylation with Shc complex formation. *A*, coprecipitation of endogenous EGF receptor and p185^{neu} with Shc following G$_i$-coupled receptor or EGF receptor stimulation. Serum-starved cells, transiently transfected with empty pRK5 vector or α2A AR, were stimulated for 2 min with UK14304 (*UK*), LPA, or EGF as indicated. Immunoprecipitates of Shc from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-EGF receptor (*top panel*), anti-p185^{neu} (*HER2, center panel*), or anti-Shc (*bottom panel*) as described. The position of EGF receptor, p185^{neu}, and Shc isoforms are as indicated. *B*, tyrosine phosphorylation of endogenous EGF receptor and p185^{neu} following G$_i$-coupled receptor or EGF receptor stimulation. Serum-starved cells, transiently transfected with empty pRK5 vector or α2A AR, were stimulated with UK14304, LPA, or EGF as indicated. Immunoprecipitates of EGF receptor (*top panel*), p185^{neu} (*center panel*), or Shc (*bottom panel*) from RIPA/SDS buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine as described. *C*, coprecipitation of Grb2, Shc, and c-Src with endogenous EGF receptor following LPA receptor or EGF receptor stimulation. Serum-starved cells were stimulated for 2 min with LPA or EGF as indicated. Immunoprecipitates of EGF receptor from nondenatured RIPA buffer lysates were resolved by SDS-PAGE and immunoblotted with anti-EGF receptor, anti-Grb2, anti-Shc, or anti-c-Src as described. The position of EGF receptor, Grb2, Shc isoforms, and c-Src are as indicated. *NS*, nonstimulated.

ulated cell lysates, suggesting that activation of the G$_i$-coupled receptor results in association of EGF receptor, c-Src, Shc, and Grb2 in a multiprotein complex.

Src Family Kinase Activity Is Required for Both G$_i$-coupled Receptor and Gβγ Subunit-mediated Tyrosine Phosphorylation of EGF Receptor and Shc—G$_i$-coupled receptor-mediated increases in EGF receptor phosphotyrosine might result from ligand-independent activation of the receptor tyrosine kinase, phosphorylation by an activated nonreceptor tyrosine kinase, or inhibition of a phosphotyrosine phosphatase. To distinguish between these alternative mechanisms, we employed a monoclonal anti-EGF receptor antibody specific for autophosphorylated EGF receptor. This antibody selectively recognizes activated EGF receptor via an epitope distal to amino acid 1052 (26), which is distinct from the major *in vitro* c-Src phosphorylation sites (33). As shown in Fig. 3 (*A* and *B*), antiphosphotyrosine immunoblots of EGF receptor immunoprecipitated from EGF-stimulated cells, from cells transiently expressing Y530F p60^{c-src}, and from cells in which phosphotyrosine phosphatase activity is inhibited by incubation with sodium orthovanadate, each exhibit increased total receptor phosphorylation. Identical immunoblots probed with the anti-activated EGF receptor antibody give increased signals from EGF-stimulated and sodium orthovanadate-treated cells but not from Y530F p60^{c-src}-transfected cells. Thus, the anti-activated EGF receptor antibody is able to discriminate between increased

autophosphorylation resulting from activation of the intrinsic tyrosine kinase activity of the EGF receptor or inhibition of a phosphotyrosine phosphatase *versus* phosphorylation of the EGF receptor mediated by the c-Src nonreceptor tyrosine kinase. As shown, this antibody does not detect EGF receptor autophosphorylation following stimulation of LPA or α2A AR receptors, despite a 3–5-fold increase in total EGF receptor phosphotyrosine, suggesting that the increase in receptor tyrosine phosphorylation does not reflect activation of the intrinsic tyrosine kinase.

Because expression of activated mutant c-Src is sufficient to mediate EGF receptor phosphorylation in the absence of ligand, we tested the hypothesis that G$_i$-coupled receptor-mediated activation of Src family kinases can account for the observed tyrosine phosphorylation of Shc and EGF receptor. To inhibit endogenous Src family kinases, cells were transiently transfected with cDNA encoding either Csk or a kinase-inactive dominant negative mutant c-Src (K298M p60^{c-src}; Ref. 34). Csk is a cytoplasmic tyrosine protein kinase (35) that inactivates Src family kinases by phosphorylating the regulatory carboxyl-terminal tyrosine residue. Csk overexpression has been shown to impair G protein-coupled receptor-mediated MAP kinase activation in COS-7 cells (19) and c-*fos* transcription in rat glomerular mesangial cells (25). As shown in Fig. 4 (*A* and *B*), coexpression of either Csk or K298M p60^{c-src} markedly inhibits Gβ1γ2 subunit-, α2A AR-, and LPA receptor-

A EGF-R immunoprecipitate
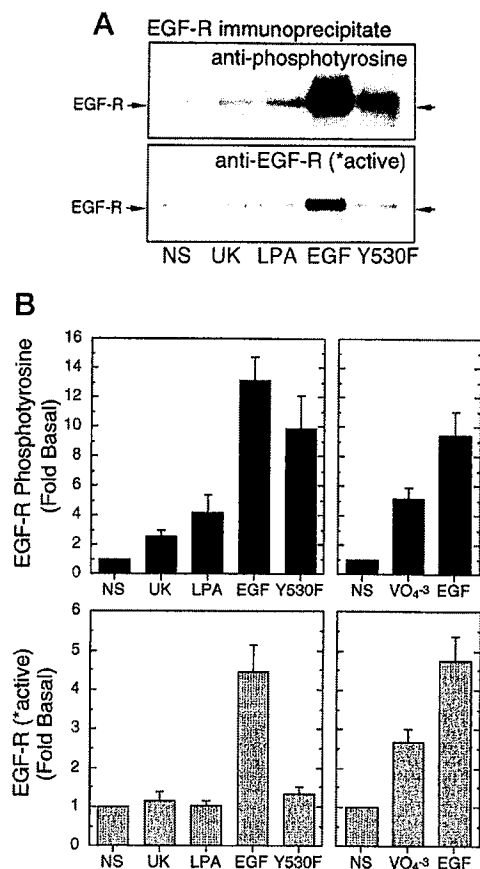

NS UK LPA EGF Y530F

B

FIG. 3. **Discrimination between Src kinase-mediated EGF receptor phosphorylation and EGF receptor autophosphorylation using anti-activated EGF receptor antibody.** A, comparison of anti-phosphotyrosine immunoblots of endogenous EGF receptor with anti-activated EGF receptor immunoblots following G_i-coupled receptor and EGF receptor stimulation, expression of Y530F, and inhibition of phosphotyrosine phosphatase activity. Serum-starved cells, transiently transfected with empty pRK5 vector, α2A AR, or Y530F p60^{c-src} were stimulated for 2 min with UK14304 (*UK*), LPA, or EGF or incubated for 20 min in the presence of sodium orthovanadate (VO$_4^{-3}$, 10 μM) as indicated. Duplicate immunoprecipitates of EGF receptor from RIPA/SDS buffer lysates were resolved by SDS-PAGE and immunoblotted with either anti-phosphotyrosine (*upper panel*) or anti-activated EGF receptor antibody (*lower panel*) as described. B, quantitation of anti-phosphotyrosine immunoblots of EGF receptor and anti-activated EGF receptor immunoblots. UK14304-, LPA-, EGF-, sodium orthovanadate-, and Y530F p60^{c-src}-stimulated EGF receptor total tyrosine phosphorylation and autophosphorylation were determined as described. Autoradiographs were quantified by scanning laser densitometry, and the data were presented as fold increase over nonstimulated or empty pRK5 vector transfected controls. The data shown represent the means ± S.E. for three separate experiments. *NS*, nonstimulated.

mediated tyrosine phosphorylation of both Shc and EGF receptor. EGF-stimulated Shc and EGF receptor phosphorylation were less dramatically effected. Shc and EGF receptor phosphorylation mediated by Y530F, which is not a substrate for Csk, is not significantly attenuated by Csk overexpression.

c-Src SH2 Domain GST Fusion Proteins Bind EGF Receptor Following G_i-coupled Receptor Activation—To determine whether G_i-coupled receptor-stimulated EGF receptor phosphorylation can induce binding of Src kinases directly to the EGF receptor, GST-fusion proteins containing either the c-Src SH2, SH3, or SH2-SH3 domains (27) were assayed for the ability to precipitate phosphorylated EGF receptor from lysates of LPA-stimulated cells. As shown in Fig. 5A, the c-Src SH2 and SH2-SH3 domain GST fusion proteins but not the c-Src SH3 domain GST fusion protein precipitate a 180-kDa tyrosine

phosphoprotein band that increases in intensity following LPA or EGF receptor stimulation. Immunoblots of EGF receptor in c-Src GST SH2 domain precipitates, shown in Fig. 5B, reveal increased association of EGF receptor with the c-Src SH2 domain following LPA or EGF stimulation, suggesting that LPA-stimulated tyrosine phosphorylation of the EGF receptor is responsible for recognition of EGF receptor by the c-Src SH2 domain.

In vitro mapping of phosphorylation sites on the EGF receptor has suggested that phosphorylation of the putative c-Src SH2 domain recognition site (Tyr^{891}) is mediated by the c-Src kinase rather than the intrinsic receptor tyrosine kinase (33). To determine if phosphorylation of this site is mediated by endogenous Src kinases in the intact cell following G_i-coupled receptor stimulation, we tested the effect of Csk overexpression on LPA-stimulated phosphorylation of the c-Src SH2 domain binding site. As shown in Fig. 5C, the ability of the c-Src SH2 domain GST fusion protein to precipitate EGF receptor following LPA stimulation is markedly attenuated in Csk-expressing cells. Because Src kinases also mediate phosphorylation of this site following receptor tyrosine kinase activation, EGF receptor precipitation by the c-Src SH2 domain GST fusion protein following stimulation with EGF is also significantly attenuated. These data suggest that G_i-coupled receptor stimulation results in both c-Src mediated phosphorylation of the EGF receptor and SH2 domain-dependent c-Src-EGF receptor complex formation.

DISCUSSION

Several lines of evidence suggest that Src family kinases play a key role in the transduction of mitogenic signals by G protein-coupled receptors. Pertussis toxin-sensitive activation of the Src family kinases Src, Fyn, Yes, and Lyn (18–21) in various cell types has been reported, and inhibition of Src family kinases has been shown to block G protein-coupled receptor-mediated Ras and phospholipase C-γ1 activation, MAP kinase activation, and c-*fos* transcription (19, 22–24). Our data demonstrate that in COS-7 cells, G_i-coupled receptor-stimulated tyrosine phosphorylation of the EGF receptor results in formation of a complex between the membrane-associated EGF receptor and the cytosolic adapter proteins Shc and Grb2, thus providing a scaffold for the assembly of a mitogenic signaling complex. The G_i-coupled receptor effects can be mimicked by cellular overexpression of Gβγ subunits, suggesting that the process is Gβγ subunit-mediated. Because inhibition of endogenous Src kinases blocks both G protein-coupled receptor-mediated EGF receptor phosphorylation and binding of the EGF receptor to the c-Src SH2 domain, the data also suggest that Src family kinases directly associate with and phosphorylate the EGF receptor following G_i-coupled receptor stimulation.

Fig. 6 depicts a model of Gβγ subunit-mediated, Ras-dependent MAP kinase activation that is consistent with these data. Gβγ subunit-dependent activation of endogenous Src family nonreceptor tyrosine kinases is an early event following G_i-coupled receptor stimulation (19). Once activated, the Src kinases mediate phosphorylation of several intracellular targets, including receptor tyrosine kinases, adapter proteins such as Shc and insulin receptor substrate-1, and possibly cytoskeletally associated Src substrates such as focal adhesion kinase and paxillin. Once phosphorylated, membrane-associated proteins such as the receptor tyrosine kinases and focal adhesion kinase would provide docking sites for the SH2 domains of the Shc and Grb2 adapter molecules, resulting in the recruitment of Ras guanine nucleotide exchange factors, and potentially of other components of the mitogenic signaling complex, to the plasma membrane. The ensuing activation of Ras would recruit the Raf kinase to the membrane and initiate the phosphoryla-

A Shc immunoprecipitate

B EGF-R immunoprecipitate

C
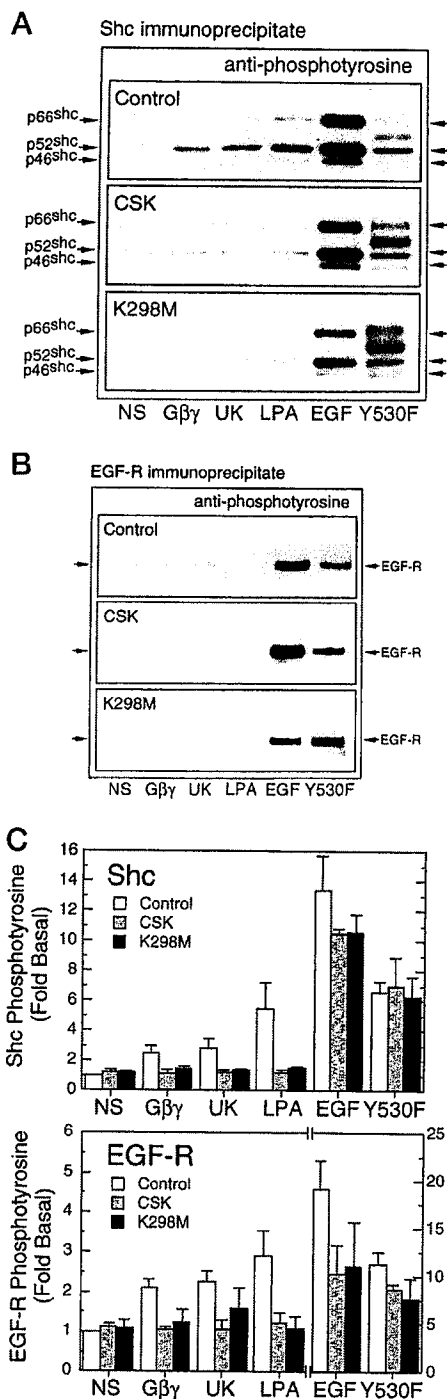
FIG. 4. **Effect of Csk and K298M p60^{c-src} expression on G$_i$-coupled receptor-mediated Shc and EGF receptor tyrosine phosphorylation.** *A*, Immunoblots of Shc phosphotyrosine following α2A AR, LPA or EGF receptor stimulation and Gβγ subunit or Y530F p60^{c-src} expression. Cells were transiently cotransfected with empty vector (*Control*) or expression plasmid encoding Csk or K298M p60^{c-src}, plus empty pRK5 vector, Gβ1 and Gγ2, α2A AR, or Y530F p60^{c-src}. Serum-starved cells were stimulated for 2 min with UK14304 (*UK*), LPA, or EGF as indicated, and immunoprecipitates of Shc from RIPA/SDS buffer lysates were immunoblotted with anti-phosphotyrosine as described. The position of tyrosine phosphorylated Shc isoforms are as indicated. *B*, immunoblots of EGF receptor phosphotyrosine following α2A AR, LPA, or EGF receptor stimulation and Gβγ subunit or Y530F p60^{c-src} expression. Serum-starved, transiently cotransfected cells were stimulated as described and immunoprecipitates of EGF receptor from RIPA/SDS buffer lysates were immunoblotted with anti-phosphotyrosine as described. The position of tyrosine phosphorylated EGF receptor is as indicated. *C*, quantitation of the effects of Csk and K298M

A anti-phosphotyrosine

B anti-EGF-R

C
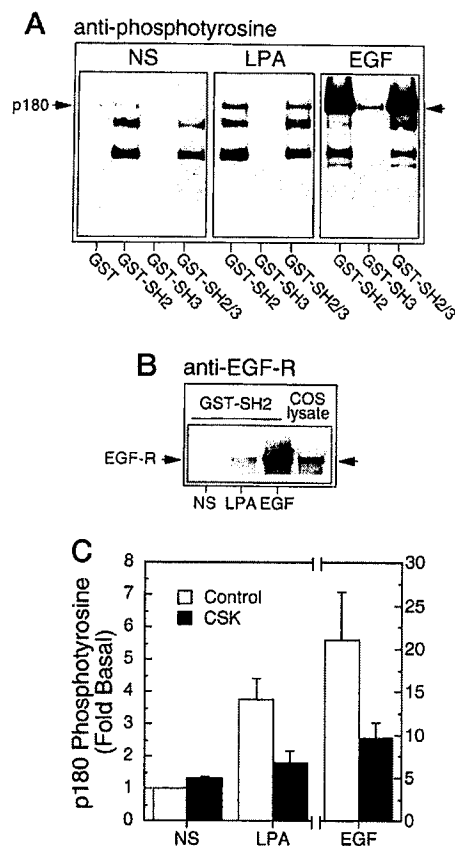
FIG. 5. **Src kinase-dependent association of EGF receptor with c-Src SH2 domain GST fusion proteins.** *A*, association of p180 with c-Src SH2 domain and SH2-SH3 domain GST fusion proteins following LPA and EGF receptor stimulation. RIPA/SDS lysates of serum-starved cells, stimulated for 2 min with LPA or EGF, were incubated with GST-Sepharose (*GST*) or GST-Src SH2, SH3, or SH2-SH3-Sepharose as described. Precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine monoclonal antibody. The position of the c-Src SH2 domain binding p180 protein is as indicated. *B*, association of EGF receptor with the c-Src SH2 domain GST fusion protein following LPA and EGF receptor stimulation. RIPA/SDS lysates of stimulated serum-starved cells were incubated with GST-Src SH2 sepharose as described. Precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-EGF receptor. The position of the EGF receptor from GST-Src SH2 Sepharose precipitates and COS-7 whole cell lysates is as indicated. *C*, quantitation of the effect of Csk expression on LPA- and EGF-stimulated association of EGF receptor with the c-Src SH2 domain GST fusion protein. Cells were transiently cotransfected with empty vector (*Control*) or expression plasmid encoding Csk (*CSK*), and LPA- and EGF-stimulated association of EGF receptor with the c-Src SH2 domain GST fusion protein was determined. Autoradiographs were quantified by scanning laser densitometry, and the data were presented as fold increase over nonstimulated or empty pRK5 vector transfected controls. The data shown represent the means ± S.E. for three separate experiments. *NS*, nonstimulated.

tion cascade leading to MAP kinase activation.

Depending upon cell type, the G protein-coupled receptors for angiotensin II, LPA, and α-thrombin have been shown to stimulate ligand-independent tyrosine phosphorylation of PDGF receptor (16), insulin-like growth factor-1 receptor β

p60^{c-src} coexpression on Gβγ subunit-, α2A AR-, LPA-, EGF-, and Y530F p60^{c-src}-stimulated Shc and EGF receptor tyrosine phosphorylation. Shc and EGF receptor phosphotyrosine were determined as described following α2A AR, LPA, or EGF receptor stimulation and Gβγ subunit or Y530F p60^{c-src} expression. Autoradiographs were quantified by scanning laser densitometry, and the data were presented as fold increase over nonstimulated or empty pRK5 vector transfected controls. The data shown represent the means ± S.E. for three to five separate experiments. *NS*, nonstimulated.
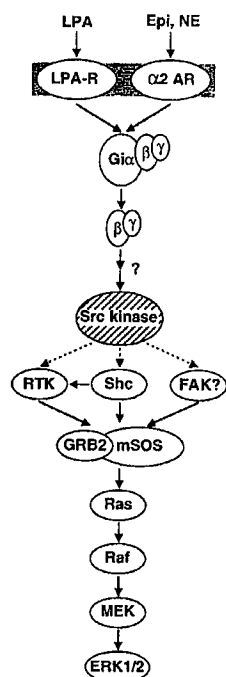
FIG. 6. **Model of G$_i$-coupled receptor-mediated, Ras-dependent MAP kinase activation in COS-7 cells.** Activation of Src family kinases following release of Gβγ subunits from pertussis toxin-sensitive heterotrimeric G proteins precedes tyrosine phosphorylation of several putative scaffolding molecules, such as receptor tyrosine kinases (*RTK*) and focal adhesion kinase, leading to the phosphotyroine-dependent, SH2 domain-mediated recruitment of Ras guanine nucleotide exchange factor (*mSos*) to the plasma membrane. The subsequent activation of Ras initiates the Raf, MAP kinase/*erk* kinase, MAP kinase phosphorylation cascade that leads to MAP kinase (*ERK 1/2*) activation.

subunit (14), EGF receptor, and p185^{neu} (17). The finding that several receptor tyrosine kinases undergo G protein-coupled receptor-mediated phosphorylation suggests the existence of a common mechanism that is not receptor tyrosine kinase-specific, such as activation of a nonreceptor tyrosine kinase or inhibition of a phosphotyrosine phosphatase. Our data, demonstrating inhibition of G$_i$-coupled receptor-mediated tyrosine phosphorylation of the EGF receptor by specific inhibitors of Src family kinases, support the hypothesis that activation of Src kinases can account for the observed receptor tyrosine kinase phosphorylation.

The role of the intrinsic tyrosine kinase activity of receptor tyrosine kinases in G$_i$-coupled receptor-mediated tyrosine phosphorylation is unclear. Daub *et al.* (17) have reported that inhibition of EGF receptor function in Rat1 cells, by either an EGF receptor-selective tyrphostin, AG1478, or expression of a dominant negative mutant EGF receptor, blocks endothelin-1, LPA, and α-thrombin receptor-mediated EGF receptor/HER2 phosphorylation and MAP kinase activation. They conclude that a ligand-independent transactivation of the EGF receptor/HER2 tyrosine kinase is responsible for G protein-coupled receptor-mediated tyrosine phosphorylation. Our data suggest that activation of Src family nonreceptor tyrosine kinases by G$_i$-coupled receptors can account for tyrosine phosphorylation of both the EGF receptor and the Shc adapter protein in COS-7 cells. The finding that inhibition of endogenous Src kinase activity blocks G$_i$-coupled receptor-mediated EGF receptor phosphorylation suggests that Src kinase activation precedes receptor tyrosine kinase phosphorylation but does not preclude the possibility that Src-mediated phosphorylation modulates the activity of the receptor tyrosine kinase. However, using a monoclonal antibody that can discriminate between c-Src-me-

diated phosphorylation and EGF receptor autophosphorylation, we have been unable to detect increased EGF receptor autophosphorylation following either overexpression of Y530F p60^{c-src} or G$_i$-coupled receptor stimulation.

The mechanism whereby effectors of activated G protein-coupled receptors stimulate Src family kinases is unknown. Stimulation of phosphatidylinositol 3-kinase activity may play a role in Ras-dependent MAP kinase activation in some cells. Gβγ subunit-mediated PI3K activity has been described in neutrophils and platelets (36, 37), a Gβγ subunit-regulated isoform of p110 PI3K has been cloned, and Gβγ subunits may contribute to the regulation of the conventional p85/p110 PI3K (38). We have previously reported that G$_i$-coupled receptor- and Gβγ subunit-mediated MAP kinase activation in COS-7 and CHO cells is sensitive to the PI3K inhibitors wortmannin and LY294002 and to expression of a dominant negative form of the p85 regulatory subunit of PI3K (39). Interestingly, MAP kinase activation by transiently expressed Y530F p60^{c-src},[2] mSos, and constitutively activated mutants of Ras and MAP kinase/*erk* kinase (39) is wortmannin-insensitive, suggesting that the PI3K-dependent step in the pathway may lie upstream of Src kinase activation. The recent report that the c-Src SH2 domain can bind with high affinity to phosphatidylinositol 3,4,5-trisphosphate, the product of PI3K (40), may provide an explanation for this phenomenon.

Interaction between Src kinases and novel Gβγ subunit-regulated nonreceptor tyrosine kinases might also contribute to the regulation of Src kinase activity. In neuronal cells, G$_q$-coupled receptors have been shown to stimulate the Ca^{2+} and protein kinase C dependent tyrosine protein kinase, PYK2 (41). PYK2 is a member of the focal adhesion kinase family of integrin receptor-associated tyrosine kinases and like p125^{FAK} (42) can complex with activated c-Src upon stimulation (43). However, phospholipase C activation and Ca^{2+} mobilization are apparently unable to account for G protein-coupled receptor-mediated tyrosine phosphorylation in many noneuronal cells (4, 44, 45). Bruton's tyrosine kinase (Btk) and Tsk, two members of a family of pleckstrin homology domain-containing tyrosine protein kinases that includes Btk, Itk, Tsk and Tec A, are reportedly regulated by Gβγ subunits (46). In hematopoietic cells, Btk interacts with the Src family kinases Fyn, Lyn, and Hck (47), and Src-Btk interaction is associated with Btk autoactivation (48). This is unlikely to be a general mechanism for G protein-coupled receptor regulation of Src kinases, however, because the pleckstrin homology domain-containing tyrosine kinases appear to have limited tissue distribution and are not known to be involved in the regulation of Ras.

The data presented in this report suggest that both Src family kinases and receptor tyrosine kinases play central roles in directing the assembly of membrane-associated mitogenic signaling complexes in response to G$_i$-coupled receptor activation in some cells. An understanding of the mechanisms whereby G protein-coupled receptors regulate tyrosine protein phosphorylation and of the basis for cross-talk between G protein-coupled receptor and receptor tyrosine kinase signaling pathways may ultimately provide strategies for selective activation or inhibition of cellular proliferation.

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Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival

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Adhesion of human primary skin fibroblasts and ECV304 endothelial cells to immobilized matrix proteins, β1 or αv integrin antibodies stimulates tyrosine phosphorylation of the epidermal growth factor (EGF) receptor. This tyrosine phosphorylation is transiently induced, reaching maximal levels 30 min after adhesion, and it occurs in the absence of receptor ligands. Similar results were observed with EGF receptor-transfected NIH-3T3 cells. Use of a kinase-negative EGF receptor mutant demonstrates that the integrin-stimulated tyrosine phosphorylation is due to activation of the receptor's intrinsic kinase activity. Integrin-mediated EGF receptor activation leads to Erk-1/MAP kinase induction, as shown by treatment with the specific inhibitor tyrphostin AG1478 and by expression of a dominant-negative EGF receptor mutant. EGF receptor and Erk-1/MAP kinase activation by integrins does not lead *per se* to cell proliferation, but is important for entry into S phase in response to EGF or serum. EGF receptor activation is also required for extracellular matrix-mediated cell survival. Adhesion-dependent MAP kinase activation and survival are regulated through EGF receptor activation in cells expressing this molecule above a threshold level (5×10^3 receptors per cell). These results demonstrate that integrin-dependent EGF receptor activation is a novel signaling mechanism involved in cell survival and proliferation in response to extracellular matrix.
Keywords: adhesion-dependent cell survival/cell–matrix interaction/EGF receptor/integrin signaling/MAP kinase

Introduction

Integrins are cell surface adhesive receptors formed by α and β subunits, which bind to extracellular matrix proteins. Integrin-mediated adhesion stimulates multiple signaling pathways which modulate actin cytoskeleton organization, cell motility, cell growth and the ability of cells to escape from apoptosis. Integrin-dependent signaling pathways include Ca^{2+} influx, cytoplasmic alkalinization, potassium channel activation and tyrosine phosphorylation of cyto-

plasmic proteins (reviewed in Clark and Brugge, 1995). Moreover, integrin-mediated adhesion triggers activation of the mitogen-activated protein (MAP) kinases ERK-1 and ERK-2 (Chen *et al.*, 1994; reviewed in Assoian, 1997; Giancotti, 1997; Howe *et al.*, 1998).

Although many integrin-dependent signaling pathways have been described extensively, the molecular mechanisms by which integrins are able to trigger these events are still poorly defined. Since the cytoplasmic domains of both α and β integrin subunits are short and devoid of enzymatic activity, it is likely that interactions with transducing molecules have to take place in order to promote intracellular signaling. A potential candidate as a transducing element is represented by the tyrosine kinase p125Fak (Hanks *et al.*, 1992; Schaller *et al.*, 1992), which is located in focal adhesions and can interact with both structural cytoskeletal elements and signaling molecules. The N-terminal domain of p125Fak binds *in vitro* the cytoplasmic domain of β1 and β3 integrin subunits, while its C-terminal domain binds SH2 and SH3 domains of several proteins involved in focal adhesion assembly and signal transduction (for a review, see Malik and Parsons, 1996). Following integrin-mediated phosphorylation, Tyr397 becomes a high-affinity binding site for the SH2 domain of c-Src (Schaller *et al.*, 1994), and this interaction is important in the regulation of cell motility (Cary *et al.*, 1996). A role for p125Fak and associated proteins in focal adhesion turnover and cell motility is also indicated by the use of the dominant-negative form of p125Fak (Richardson and Parsons, 1996) and by p125Fak gene inactivation (Ilic *et al.*, 1995). It has also been proposed that phosphorylated p125Fak interacts with the adaptor molecule Grb-2, leading to MAP kinase activation (Schlaepfer and Hunter, 1997).

The assembly of a transduction complex can also involve transmembrane proteins that co-operate with integrins to activate signaling pathways. In particular, it has been shown that caveolin, a membrane protein that links a variety of cell surface receptors to intracellular signaling pathways, can interact with integrins. Following cell–matrix adhesion, integrin–caveolin complexes associate with tyrosine-phosphorylated Shc, which, in turn, interacts with the Grb2–Sos complex leading to activation of the Ras–MAP kinase cascade (Wary *et al.*, 1996). In addition, integrins can associate with proteins belonging to the Tetraspan family (CD9, CD63 and CD81) to modulate intracellular signaling (Berditchevski *et al.*, 1997).

In addition to these molecules, growth factor receptors are candidates to co-operate with integrins in assembling the transduction machinery. Evidence for the formation of integrin and growth factor receptor macromolecular complexes has been suggested by co-clustering and immunofluorescence experiments (Plopper *et al.*, 1995; Miyamoto *et al.*, 1996; Sundberg and Rubin, 1996; Jones

et al., 1997) as well as by direct co-precipitation (Falcioni *et al.*, 1997; Schneller *et al.*, 1997). Schneller *et al.* (1997) showed for the first time that activation by its specific ligand leads to association of the platelet-derived growth factor (PDGF) β receptor with the αvβ3 integrin.

In the present study, we demonstrate that integrins can utilize the epidermal growth factor (EGF) receptor as a transducing element in the matrix-induced signaling pathways. We show in fact that integrins can induce EGF receptor tyrosine phosphorylation in the absence of EGF receptor ligands, leading to activation of a typical EGF receptor pathway that involves Shc phosphorylation and MAP kinase activation. In addition, we show that integrin-dependent EGF receptor activation is important in anchorage-dependent cell survival.

Results

Integrin-mediated adhesion induces tyrosine phosphorylation of the EGF receptor

The plating of human primary skin fibroblasts N6 and human endothelial cells ECV304 on matrix proteins in serum-free medium stimulates rapid tyrosine phosphorylation of 170 and 125/130 kDa proteins (Figure 1A, lanes 1–4). The 125/130 kDa proteins were identified as p125Fak and p130Cas, as previously described (Burridge *et al.*, 1992; Defilippi *et al.*, 1994; Nojima *et al.*, 1995). To identify the 170 kDa protein, we used a panel of antibodies and showed that this protein was immunoprecipitated specifically by the EGF receptor antibody 2913 (Beguinot *et al.*, 1986) (Figure 1A, lanes 5 and 6). Immunodepletion of EGF receptor from cell extracts resulted in the disappearance of the 170 kDa protein, indicating that EGF receptor is the only component of this tyrosine-phosphorylated band (Figure 1A, lanes 7 and 8). Tyrosine phosphorylation of EGF receptor in response to adhesion was also observed by plating NIH-3T3 cells transfected with the EGF receptor (Velu *et al.*, 1989) on fibronectin-coated dishes (Figure 1A, lanes 9 and 10).

In order to test whether tyrosine phosphorylation of the EGF receptor during cell–matrix adhesion is induced specifically by integrins, we plated ECV304 cells on dishes coated with monoclonal antibodies to β1 and αv integrin subunits or with poly-L-lysine. While tyrosine phosphorylation of the EGF receptor was induced similarly in cells plated on both integrin antibodies, cells plated on poly-L-lysine did not display significant levels of receptor phosphorylation (Figure 1B). Moreover, tyrosine phosphorylation of the EGF receptor was also stimulated by antibody-induced β1 or β3 integrin clustering in ECV304 cells kept in suspension, indicating that integrin oligomerization by antibodies is sufficient to induce this event (data not shown).

To exclude the possibility that EGF receptor phosphorylation induced by integrin-mediated adhesion was dependent on autocrine production of its ligands, EGF receptor (EGFR)-transfected NIH-3T3 cells were treated with concentrated supernatants of both N6 and ECV304 cells, and analyzed for tyrosine phosphorylation of the EGF receptor. Neither N6- nor ECV304-conditioned medium induced any receptor phosphorylation (data not shown). Furthermore, no mRNAs for EGF or transforming growth factor-α (TGF-α) were detected by RT–PCR experiments in N6
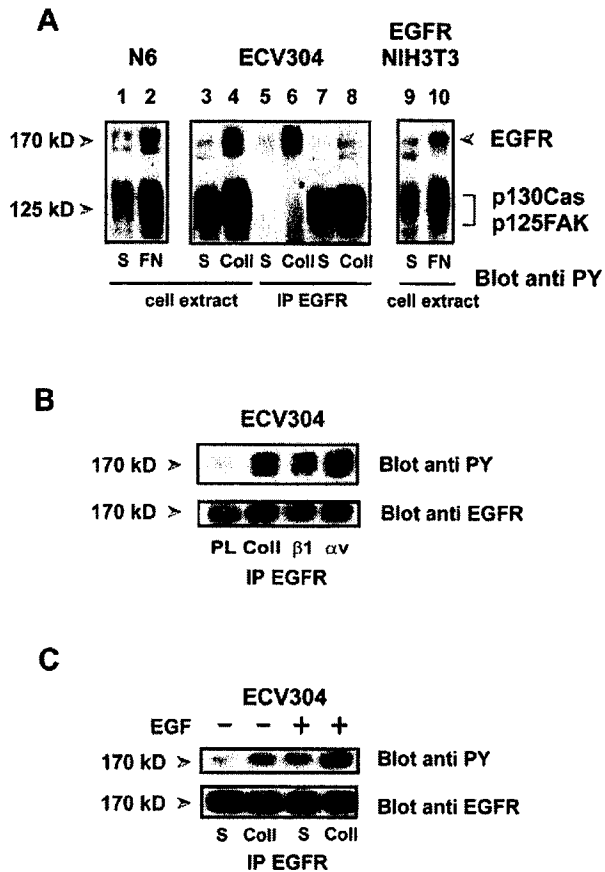
Fig. 1. Analysis of adhesion-dependent EGF receptor tyrosine phosphorylation. (**A**) N6 primary human skin fibroblasts (lanes 1 and 2), ECV304 endothelial cells (lanes 3–8) and EGFR-transfected NIH-3T3 cells (EGFR NIH3T3) (lanes 9 and 10) were serum starved, detached with EDTA and either kept in suspension (S) or allowed to adhere on dishes coated with 10 µg/ml fibronectin (FN), or collagen type I (Coll) for 30 min. Aliquots of 100 µg of cells extracts (lanes 1–4, 9 and 10) were run on 8% SDS–PAGE and immunoblotted with anti-phosphotyrosine (anti-PY) mAb PY20. A 1 mg aliquot of ECV304 cell extracts was immunoprecipitated with the EGF receptor polyclonal antibody 2913 (lanes 5 and 6), and supernatants, following two rounds of immunoprecipitations (lanes 7 and 8), were separated by SDS–PAGE and analyzed by Western blotting with anti-PY mAb PY20. The arrow on the right indicates the position of the EGF receptor; the bracket indicates the doublet constituted by p125Fak and p130Cas. (**B**) ECV304 cells were kept in suspension or plated on dishes coated with 10 µg/ml poly-L-lysine (PL), Coll, mAb BV7 to β1 integrin or mAb L230 to the αv integrin subunit. Cell extracts (1 mg) were immunoprecipitated with the EGF receptor polyclonal antibody 2913, resolved by SDS–PAGE and immunoblotted with anti-PY mAb PY20 (upper panel). After stripping, the filter was reprobed with polyclonal antibody EGFR1 (lower panel). (**C**) ECV304 endothelial cells were kept in suspension (S) or allowed to adhere on dishes coated with 10 µg/ml Coll for 30 min in the absence (−) or presence (+) of 15 ng/ml of EGF. Cell extracts were immunoprecipitated with the EGF receptor polyclonal antibody 2913, run on SDS–PAGE and immunoblotted with anti-PY mAb PY20 (upper panel). The filter was reprobed with polyclonal antibody EGFR1 (lower panel). Molecular weights are indicated on the left.

or ECV304 cells (data not shown). Taken together, these findings indicate that adhesion-induced EGF receptor phosphorylation is not mediated by release of receptor ligands during the adhesive process.

To compare the intensity of tyrosine phosphorylation of the EGF receptor induced by adhesion with that induced

A

ECV304

0 15' 30' 1h 2h 4h

170 kD ➤   IP EGFR

adhesion to collagen

Blot anti PY

B

ECV304

- - + + cytoD

170 kD ➤   IP EGFR

125 kD ➤   IP p125FAK

S Coll S Coll

Blot anti PY

Fig. 2. Time course of EGF receptor tyrosine phosphorylation following adhesion, and effect of cytochalasin D treatment. (A) ECV304 endothelial cells were plated on 10 µg/ml collagen type I-coated dishes for the indicated times. Cell extracts (1 mg) were immunoprecipitated with the polyclonal antibody 2913. Immunoprecipitates were run on SDS–PAGE and immunoblotted with anti-PY mAb RC20. (B) Extracts of ECV304 cells kept in suspension or allowed to adhere for 30 min on 10 µg/ml collagen type I in the absence (–) or presence (+) of 0.4 µM cytochalasin D (cytoD) were immunoprecipitated with the polyclonal antibodies EGFR1 or Fak4. Tyrosine-phosphorylated EGF receptor and p125Fak were detected with anti-PY mAb RC20. A representative experiment of three is shown.

by EGF, ECV304 cells were treated with EGF either in suspension or adherent to matrix proteins. Quantitative analysis of phosphotyrosine Western blotting indicated that adhesion-induced tyrosine phosphorylation of the EGF receptor is 5-fold less than that induced by a saturating dose of EGF (15 ng/ml) (Figure 1C, lanes 2 and 4) and similar to that obtained with 1 ng/ml EGF (data not shown). Therefore, EGF receptor tyrosine phosphorylation induced by adhesion represents a partial receptor activation, which can be increased further by addition of subsaturating concentration of EGF. In addition, the EGF receptor is phosphorylated more efficiently by the same doses of EGF in adherent cells than in cells in suspension (Figure 1D, lanes 3 and 4).

Integrin-mediated tyrosine phosphorylation of EGF receptor is transient and independent of actin cytoskeleton organization

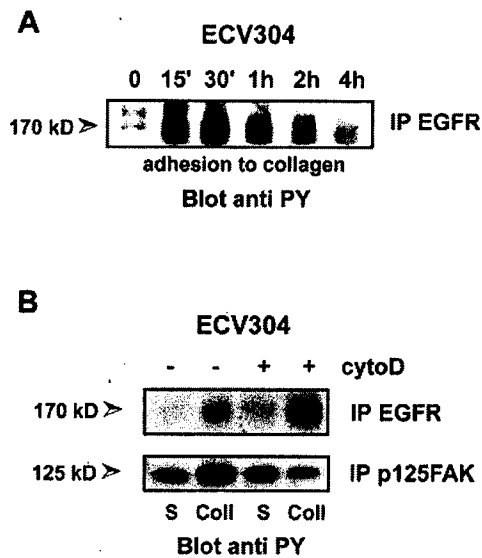In ECV304 cells plated on collagen type I, tyrosine phosphorylation of the EGF receptor was maximal 30 min after plating and then decreased, reaching basal levels within 4 h (Figure 2A). Phosphorylation of EGF receptor following adhesion is therefore a transient phenomenon.

Actin cytoskeleton integrity has been shown to be a major requirement in many integrin-mediated signaling events (Clark and Brugge, 1995). Therefore, ECV304 cells were plated on collagen type I in the presence of 0.4 µM cytochalasin D, a known inhibitor of actin polymerization. This treatment inhibited spreading but not adhesion on collagen. Phosphotyrosine Western blotting showed that cytochalasin D did not influence

A

ECV304   **EGFR NIH3T3**

IP: + - β1 integrin   IP: + - EGFR
     - + β2 microglobulin

◁ EGFR   ◁ β1 integrin
                ◁ pre β1

Blot anti EGFR   **Blot anti β1**

B

ECV304

S Coll   S Coll

◁ EGFR

◁ β1 integrin

1 2   3 4

IP β1   IP EGFR

Blot streptavidin
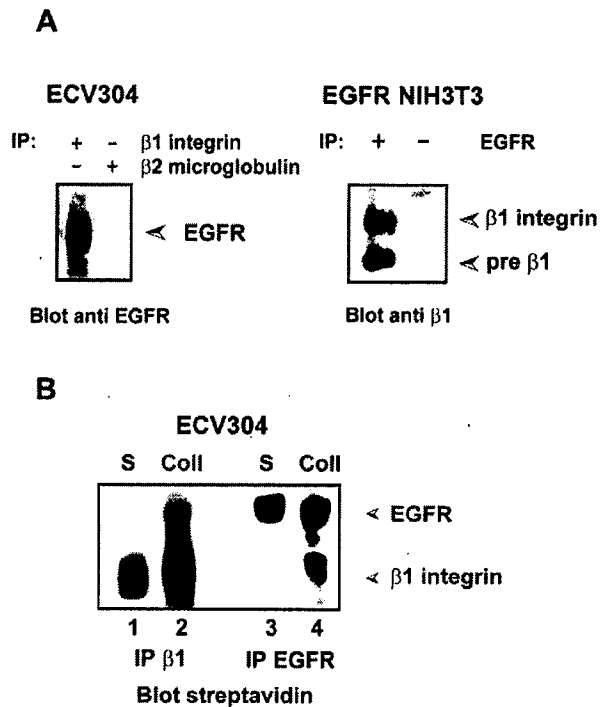
Fig. 3. Co-immunoprecipitation between EGF receptor and β1 integrin. (A) Cell extracts from ECV304 endothelial cells and EGFR-transfected NIH-3T3 cells were immunoprecipitated with mAb BV7 to the β1 integrin subunit, mAb RI.30 to human β2-microglobulin, polyclonal antibody EGFR1 or pre-immune serum (–), run on SDS–PAGE, and immunoblotted with polyclonal antibody EGFR1 or with polyclonal antibody to β1 integrin. (B) ECV304 cells kept in suspension (S) or allowed to adhere on dishes coated with 10 µg/ml collagen were cell surface biotinylated, and extracts immunoprecipitated with mAb BV7 to the β1 integrin subunit or polyclonal antibody EGFR1 were revealed with streptavidin–HRP. The positions of the EGF receptor and β1 integrin are shown on the right.

adhesion-induced tyrosine phosphorylation of the receptor (Figure 2B, upper panel), while it dramatically reduced that of p125Fak (Figure 2B, lower panel), in agreement with previous observations (Lipfert *et al.*, 1992; Defilippi *et al.*, 1995).

β1 integrin and EGF receptor can form a complex on the cell membrane

To investigate whether the EGF receptor and integrins associate, extracts of ECV304 cells were immuno-precipitated with β1 antibodies and blotted with EGF receptor antibodies. The EGF receptor was indeed co-immunoprecipitated with β1 integrin (Figure 3A, left panel) and the β1 integrin was detected in the anti-EGF receptor immunoprecipitate (Figure 3A, right panel), indicating that these molecules can form a complex. Immunoprecipitation of EGF receptor with β1 integrin was specific, since no co-immunoprecipitation of EGF receptor was observed with a control antibody against β2-microglobulin or pre-immune serum (Figure 3A). Co-immunoprecipitation of EGF receptor and integrins was corroborated further by using cell surface biotinylated ECV304 cells kept in suspension or plated on dishes coated with collagen. A 170 kDa band corresponding to the EGF receptor was found in the β1 immunoprecipitate from cells adherent to collagen, but not from cells kept

A

ECV304

| | S | EGF | EGF | VN | Coll | VN | Coll |

tyrphostin AG1478   -   -   +   -   -   +   +

EGFR ➤

1 2 3 4 5 6 7

EGFR ➤

Blot anti EGFR

B

EGFR NIH3T3

| | S | FN | VN |

MBP ➤

EGFR ➤

Blot anti EGFR

C

| EGFR NIH3T3 | | EGFR/K- NIH3T3 | |
| S | FN | S | FN |

◄ EGFR ➤

Blot anti PY

◄ EGFR ➤

Blot anti EGFR

| EGFR NIH3T3 | EGFR/K- NIH3T3 |
| - | + | - | + | EGF |

◄ EGFR ➤

Blot anti PY

◄ EGFR ➤

Blot anti EGFR

Fig. 4. Analysis of EGF receptor autophosphorylation. (A) A kinase assay was performed on EGF receptor immunoprecipitated from ECV304 endothelial cells kept in suspension (S) (lane 1) or allowed to adhere on either 10 μg/ml vitronectin (VN) (lanes 4 and 6) or collagen type I (Coll) (lanes 5 and 7) for 30 min in the absence (lanes 1, 4 and 5) or presence of 250 nM tyrphostin AG1478 (lanes 6 and 7). Confluent cultures were treated with 15 ng/ml EGF (lanes 2 and 3) in the absence (lane 2) or presence (lane 3) of 250 nM tyrphostin AG1478. (B) A kinase assay was performed on EGF receptor immunoprecipitated from ECV304 endothelial cells kept in suspension (S) or allowed to adhere on either 10 μg/ml FN or VN for 30 min in the presence of MBP as endogenous substrate. (C) Upper left panel: EGFR NIH-3T3 or EGFR kinase-negative (EGFR/K⁻) NIH-3T3 cells were kept in suspension (S) or allowed to adhere on 10 μg/ml FN for 30 min. Cell extracts were resolved by SDS–PAGE and immunoblotted with anti-PY mAb PY20. Upper right panel: cell extracts of EGFR NIH-3T3 or EGFR kinase-negative (EGFR/K⁻) NIH-3T3 cells stimulated with 15 ng/ml EGF (+) or left unstimulated (–) were immunoprecipitated with the EGF receptor polyc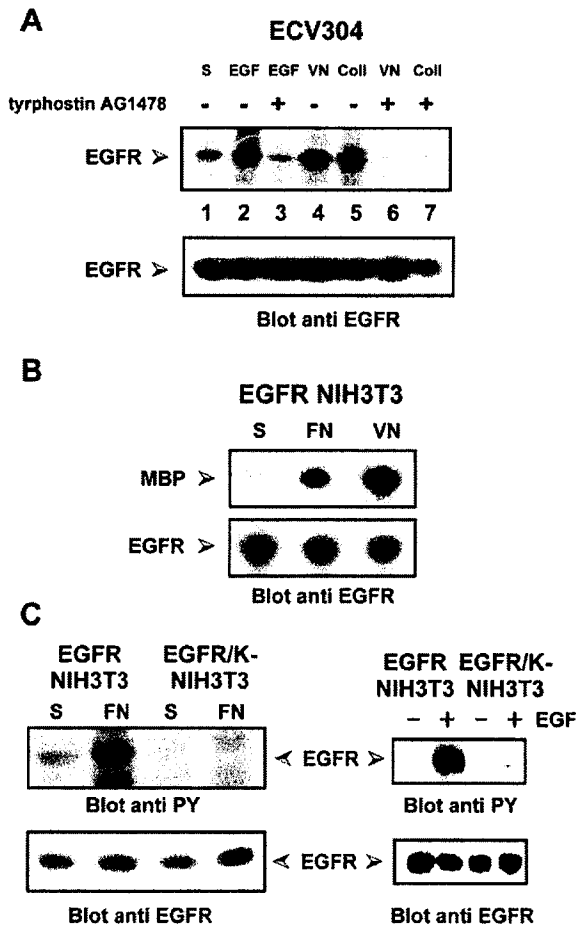lonal antibody 2913, run on SDS–PAGE and immunoblotted with anti-PY mAb PY20 (upper panel). Filters were reprobed with polyclonal antibody EGFR1 (lower panels).

in suspension (Figure 3B, lanes 1 and 2). Similarly, a band corresponding to the β1 integrin was detectable in the anti-EGF receptor immunoprecipitate (Figure 3B, lanes 3 and 4), suggesting that integrins and EGF receptor can form a complex on the cell membrane. Densitometric analysis showed that the fraction of EGF receptor associated with the β1 integrin represents approximately one-tenth of the total biotinylated cell surface EGF receptor, suggesting that the integrin-associated receptor might represent a subset of the receptor molecules.

| A EGFR NIH3T3 | | | B NIH3T3 | | |

| tyrphostin AG1478 | S | FN | FN | S | FN | FN |
| | – | – | + | – | – | + |

p52 Shc ➤

IP Shc
Blot anti PY
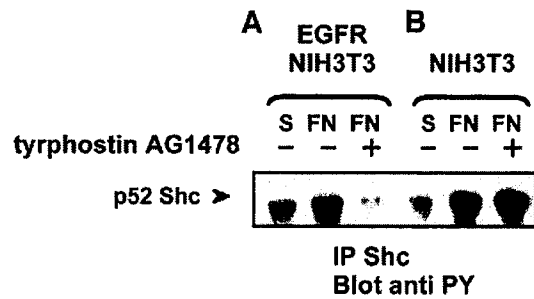
Fig. 5. Analysis of adhesion-induced Shc tyrosine phosphorylation. EGFR NIH-3T3 (A) and parental NIH-3T3 cells (B) were kept in suspension (S) or allowed to adhere on 10 μg/ml fibronectin (FN) for 30 min in the absence (–) or presence (+) of 250 nM tyrphostin AG1478. Shc protein was immunoprecipitated from cell extracts using a specific polyclonal antiserum and subsequently immunoblotted with anti-PY mAb RC20. The p52 Shc isoform is indicated by the arrow.

Integrin-mediated adhesion triggers autophosphorylation of EGF receptor

To investigate whether integrin-mediated adhesion stimulates the intrinsic receptor kinase activity, we performed kinase assays on EGF receptor immunoprecipitated from extracts of cells either plated on matrix proteins or kept in suspension. As shown in Figure 4A, EGF receptor kinase activity is 2-fold higher in cells attached to matrix than in cells in suspension (lanes 1, 4 and 5). Similar results were obtained by analyzing EGF receptor kinase activity using myelin basic protein (MBP) as exogenous substrate (Figure 4B) (Wang et al., 1991). Activation of EGF receptor kinase induced by matrix proteins was lower than that induced with 15 ng/ml EGF (Figure 4A, lane 2). To test if adhesion induces the intrinsic receptor kinase activity, we used tyrphostin AG1478, a very potent and specific inhibitor of EGF receptor kinase (Levitzki and Gazit, 1995). Tyrphostin AG1478 abolished the EGF receptor kinase activity both in matrix-adherent and in EGF-treated cells (Figure 4A, lanes 3, 6 and 7). In addition, we tested NIH-3T3 cells expressing the kinase-negative EGF receptor mutant (EGFR/K⁻ NIH-3T3) (Figure 4C, right panel) (Sorkin et al., 1992). When these cells were plated on fibronectin, neither tyrosine phosphorylation of the receptor (Figure 4C, left panel) nor kinase activity (data not shown) were detected. Therefore, we can conclude that integrin-mediated tyrosine phosphorylation of the EGF receptor requires activation of receptor kinase activity.

Adhesion-induced EGF receptor phosphorylation leads to Erk-1/MAP kinase activation

The adaptor Shc is a well-known downstream effector of the EGF receptor pathway (Pelicci et al., 1992), leading to Erk-1/MAP kinase activation. We tested whether integrin-dependent EGF receptor phosphorylation activates this pathway. Immunoprecipitation experiments indicated that the p52 Shc isoform was tyrosine phosphorylated following adhesion of EGFR-transfected NIH-3T3 cells to fibronectin. Adhesion-induced Shc tyrosine phosphorylation was abolished by treatment with tyrphostin AG1478, indicating that EGF receptor tyrosine kinase is required for this event (Figure 5A). Co-precipitation of EGF receptor and Shc in cells adherent to matrix proteins (data not shown) further confirms that integrin-mediated EGF receptor activation leads to association of Shc. In contrast,

A

EGFR NIH3T3

5   15   30   60   min

MBP ➤

Adhesion to FN

B

EGFR NIH3T3

tyrphostin AG1478

S	PL	FN	FN
-	-	-	+

MBP ➤

C

Fig. 6. Involvement of EGF receptor phosphorylation in integrin-mediated Erk-1 MAP kinase activation. Erk-1 MAP kinase activity was determined using MBP as exogenous substrate as described in Materials and methods, and phosphorylated MBP (arrow) was visualized by aut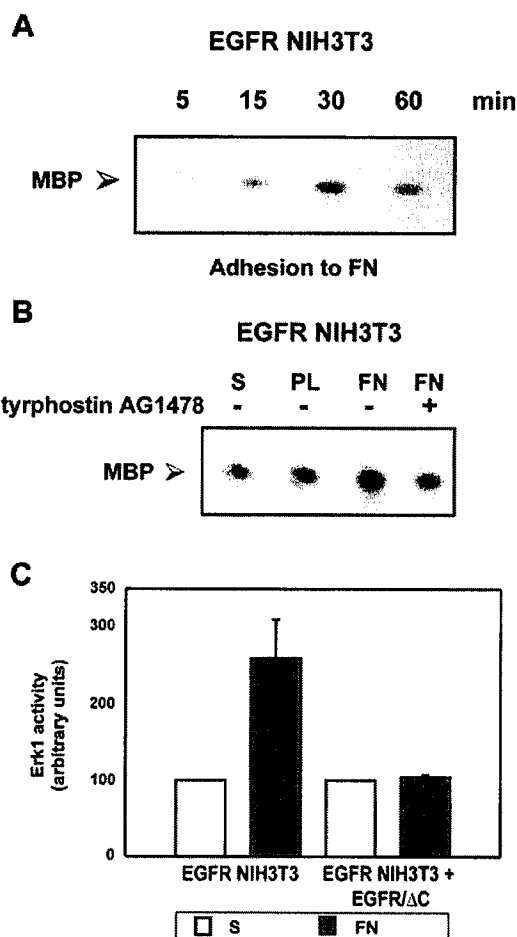oradiography after gel electrophoresis (A and B). (A) EGFR NIH-3T3 cells were plated on 10 μg/ml fibronectin (FN) for the indicated times. Note a peak of Erk-1 activity at 30 min of adhesion. (B) EGFR NIH-3T3 cells were kept in suspension (S) or plated either on 10 μg/ml poly-L-lysine (PL) or FN for 30 min in the presence (+) or absence (–) of 250 nM tyrphostin AG1478. (C) EGFR NIH-3T3 and EGFR NIH-3T3 cells co-transfected with the dominant-negative form of the EGF receptor (EGFR NIH3T3 + EGFR/ΔC) were kept in suspension (S, white bars) or plated on 10 μg/ml fibronectin (FN, gray bars). Phosphorylated MBP was quantified by densitometric analysis. Data are shown as the mean ± SE of five independent experiments.

in parental NIH-3T3 cells, which express very low levels of endogenous EGF receptors (~5×10³ molecules per cell) (Sorkin *et al.*, 1992), p52 Shc was phosphorylated following adhesion, but this phosphorylation was not reduced by tyrphostin AG1478 treatment (Figure 5B), suggesting that in these cells, Shc phosphorylation following adhesion does not depend on EGF receptor activation.

To assess activation of the Erk-1/MAP kinase pathway, EGFR-transfected NIH-3T3 cells were plated on fibronectin for different times, and Erk-1/MAP kinase activity was evaluated. Erk-1 activity was maximal after 30 min of adhesion to fibronectin, and decreased after 1 h (Figure 6A). Comparison between the time course of adhesion-induced Erk-1 activation and EGF receptor tyro-

sine phosphorylation indicates that the two processes reach maximal levels with similar kinetics (data not shown). If cells were treated with tyrphostin AG1478 specifically to inhibit EGF receptor kinase, Erk-1 activity in response to adhesion was almost completely inhibited (Figure 6B). Similarly, co-expression of a dominant-negative receptor mutant, lacking almost the entire cytoplasmic domain, EGFR/ΔC, caused a dramatic inhibition of Erk-1 activation by adhesion (Figure 6C). Thus adhesion-dependent Erk-1/MAP kinase induction requires EGF receptor activation. To confirm these results in cells naturally expressing EGF receptor, we tested Erk-1 activation in ECV304 cells by clustering β1, β3 or αv subunits by specific antibodies. Quantitative analysis showed that in these cells Erk-1 activation was reduced by tyrphostin AG1478 treatment (Figure 7A). Interestingly, in parental NIH-3T3 cells, Erk-1 was activated by adhesion, but this activation was not reduced by tyrphostin AG1478 treatment (Figure 7B). Thus, the use of both tyrphostin and a dominant-negative form of the receptor indicates that EGF receptor activation by adhesion largely contributes to the integrin signaling towards activation of the Erk-1/MAP kinase pathway in cells such as primary fibroblasts, ECV or NIH-3T3 cells transfected with the EGF receptor. All these cells express >3×10⁴ EGF receptors per cell; when EGF receptor is barely expressed, such as in parental NIH-3T3 cells (<5×10³ molecules per cell), however, Erk-1/MAP kinase activation in response to integrins is controlled predominantly by an EGF receptor-independent alternative pathway.

Since EGF is known to activate MAP kinases through its receptor, we tested whether integrin-induced EGF receptor-dependent Erk-1/MAP kinase activity can still be modulated by EGF addition in adherent cells. Fibronectin-dependent Erk-1 activation was increased 2-fold when 15 ng/ml EGF were added to EGFR-transfected NIH-3T3 cells, indicating that integrin-mediated pathways are additive to that of the natural ligand (Figure 7C).

Adhesion-induced EGF receptor activation does not induce cell proliferation, but protects cells from apoptosis

To investigate whether integrin-mediated EGF receptor activation may influence the ability of cells to progress into the cell cycle, serum-deprived EGFR-transfected NIH-3T3 cells were plated on fibronectin in the absence and presence of EGF or serum. Entry into the cell cycle was measured as the percentage of cells in G₂/M phase 24 h after plating. As shown in Table I, cells plated on fibronectin do not progress into G₂/M phase unless EGF or serum is also present. Thus, transient EGF receptor and Erk-1/MAP kinase activation in response to fibronectin does not lead to a loss of requirement for growth factors to progress into the cell cycle. Adhesion, however, is important for entry into the cell cycle, since EGF or serum do not trigger entry into G₂/M phases in cells kept in suspension (data not shown).

Many cell types undergo apoptosis when deprived of adhesion to the appropriate extracellular matrix. As shown in Figure 8, EGFR-transfected NIH-3T3 cells kept in suspension for 24 h in serum-free medium enter apoptosis, while their plating on fibronectin in the absence of growth factors protects them from programmed cell death. Tyrphostin AG1478 reduced survival, and the dominant-
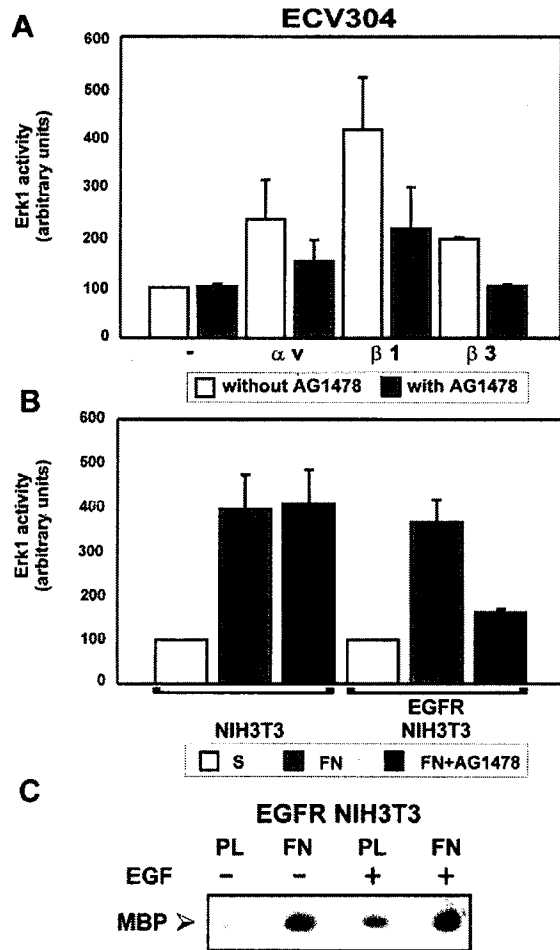
Fig. 7. Analysis of integrin-dependent Erk-1 MAP kinase activation. Immunoprecipitated Erk-1 was subjected to kinase assay and phosphorylated MBP was visualized by autoradiography after gel electrophoresis. Phosphorylated MBP was quantified by densitometric analysis. Data are shown as the mean ± SE of four independent experiments. (A) ECV304 cells kept in suspension were incubated for 30 min at 4°C in serum-free DMEM (–) containing 10 µg/ml mAbs L230 to αv, BV7 to β1, or B212 to β3 integrin subunits. Integrin clustering was induced by incubating cells with 20 µg/ml rabbit anti-mouse IgG for 30 min at 37°C, in the absence (white bars) or presence (gray bars) of 250 nM tyrphostin AG1478. (B) Parental NIH-3T3 or EGFR NIH-3T3 cells were kept in suspension (S, white bars) or seeded on 10 µg/ml fibronectin (FN) in the absence (gray bars) or presence (black bars) of 250 nM tyrphostin AG1478 for 30 min. (C) EGFR NIH-3T3 cells were plated on 10 µg/ml poly-L-lysine (PL) or fibronectin (FN) for 30 min, in the absence (–) or presence (+) of 15 ng/ml EGF.

negative EGF receptor mutant, EGFR/ΔC, completely abolished the protection induced by fibronectin. Moreover, tyrphostin AG1478 partially abolished the fibronectin-mediated survival effect in ECV304 cells, indicating that the EGF receptor is physiologically required in the cell survival response triggered by the extracellular matrix. Thus, adhesion-induced EGF receptor activation is an essential step in cell survival exerted by the extracellular matrix. To confirm further the involvement of EGF receptor in the survival pathway, we also found that parental NIH-3T3 cells are not sensitive to tyrphostin treatment, suggesting that in these cells fibronectin-mediated cell survival is regulated by pathways distinct from the

Table I. Analysis of cell cycle progression

Percentage of cells in the G_2/M phase of the cell cycle

Culture	FN	FN + EGF	FN + FCS	S + FCS
	17.98 ± 7.03	0.00 ± 0.00 4.43 ± 0.08	11.14 ± 2.55	0.00 ± 0.00

EGFR NIH-3T3 cells were serum deprived for 30 h, detached and kept in suspension (S) or plated on 10 µg/ml fibronectin (FN) for 24 h in the presence of 50 ng/ml human recombinant EGF or 10% FCS where indicated. Cells in culture were used as positive control. Cells were stained with propidium iodide, and the percentage of cells in G_2/M phase was determined by flow cytometry according to the Bio-Rad DNA-Analysis software. Data are shown as the mean ± SE of three representative independent experiments.

activation of EGF receptor (Figure 8). The addition of the MAP kinase kinase MEK inhibitor, PD98059 (Dudley et al., 1995), does not abolish the ability of EGFR-transfected NIH-3T3 cells to survive on fibronectin, indicating that the Erk-1/MAP kinase is not required for this response. In contrast, a strong inhibition of the survival of EGFR-transfected NIH-3T3 cells on fibronectin was induced by treating cells with the phosphatidylinositol-3 kinase (PtdIns-3 kinase) inhibitor wortmannin (Figure 8), while parental NIH-3T3 cells were unaffected by wortmannin treatment (not shown). Therefore, these data show that adhesion-induced EGF receptor signaling is involved in fibronectin-mediated cell survival through a pathway distinct from Erk-1/MAP kinase activation, which may involve PtdIns-3 kinase activation.

Discussion

In this study, we demonstrate that in human primary fibroblasts, endothelial cells and EGFR-transfected NIH-3T3 cells, adhesion to extracellular matrix proteins induces a transient tyrosine phosphorylation of the EGF receptor, which in turn leads to Erk-1/MAP kinase activation and to anchorage-dependent cell survival. Activation of the EGF receptor by cell–matrix adhesion is a new pathway linking integrin signaling to nuclear events.

The data reported in this work provide new evidence on the ability of integrins to co-operate directly at the membrane level with growth factor receptors in order to activate specific signaling pathways. Integrin-dependent tyrosine phosphorylation of the EGF receptor is inhibited by tyrphostin AG1478, a drug which specifically inhibits EGF receptor kinase competing for ATP (Levitzki and Gazit, 1995). The use of a kinase-negative EGF receptor confirms that tyrosine phosphorylation of EGF receptor by integrins is due to bona fide activation of intrinsic receptor kinase activity. This event is unlikely to depend on autocrine or paracrine production of EGF receptor ligands during cell adhesion. In fact, it was shown previously that NIH-3T3 cells transfected with the EGF receptor do not produce EGF receptor ligands capable of autocrine activity (Di Fiore et al., 1987; Velu et al., 1987). In addition, primary fibroblasts or ECV304 cells do not express EGF or TGF-α transcripts, and their conditioned medium is unable to stimulate EGF receptor tyrosine phosphorylation, indicating that other EGF-like ligands such as β-cellulin, heparin-binding EGF or amphiregulin
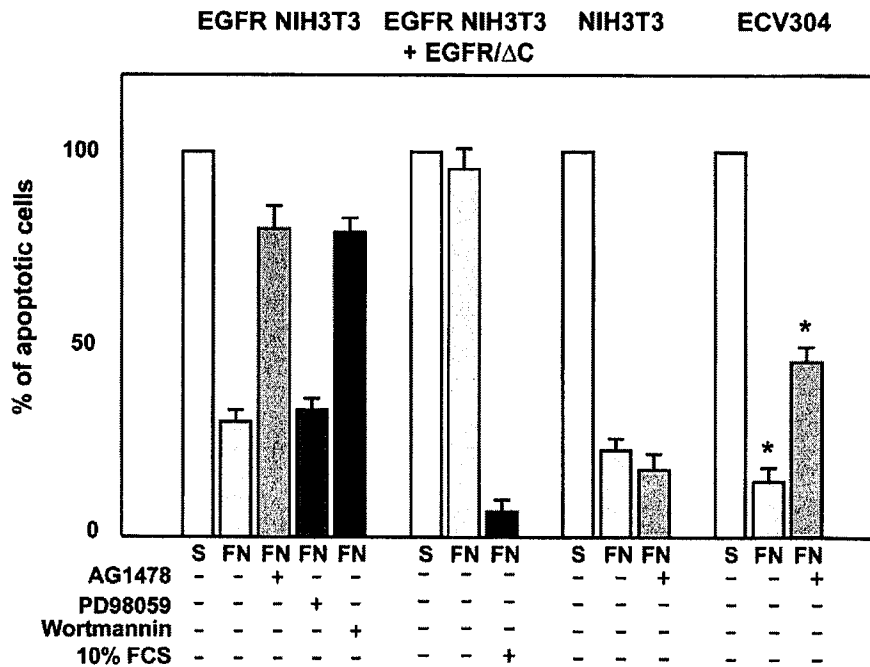
Fig. 8. Involvement of EGF receptor activation in adhesion-mediated cell survival to apoptosis. EGFR NIH-3T3, EGFR NIH-3T3 co-transfected with the dominant-negative form of the EGF receptor (EGFR NIH3T3 + EGFR/ΔC), parental NIH-3T3 and ECV304 cells were kept in suspension on polyHEMA-coated dishes (S), or allowed to adhere on 10 μg/ml fibronectin (FN) for 24 h in the absence (−) or presence (+) of 250 nM tyrphostin AG1478, 25 μM PD98059, 200 nM wortmannin or 10% FCS. Cells were stained with propidium iodide and examined by laser-scanning confocal microscope. The data were reported as the percentage of apoptotic cells over 400 nuclei scored for each sample. Data are shown as the mean ± SE of five independent experiments. *Differences at $P = 0.0001$.

(reviewed in Pinkas-Kramarski *et al.*, 1997) do not contribute to receptor activation in our cellular systems.

The data reported in this work, moreover, indicate that integrin-mediated activation of the EGF receptor is not limited to its tyrosine phosphorylation, but downstream signaling events are also generated. In particular, we have shown that integrin-induced EGF receptor phosphorylation leads to a well-defined EGF receptor pathway, consisting of Shc phosphorylation and activation of Erk-1/MAP kinase. Other mechanisms of MAP kinase activation by integrins have been reported. In particular, a subset of integrin heterodimers can associate with caveolin and promote Shc tyrosine phosphorylation, leading to Ras–MAP kinase activation (Wary *et al.*, 1996). Moreover, in cells overexpressing p125Fak, this phosphorylated molecule can recruit Grb2 and lead to activation of Ras–MAP kinase (Schlaepfer and Hunter, 1997). The activation of MAP kinase by the EGF receptor thus represents a novel mechanism by which integrins can activate this pathway. The ability of tyrphostin AG1478 and of the dominant-negative EGF receptor mutant (EGFR/ΔC) to strongly decrease or abolish this integrin-dependent signaling indicates that in our cellular system EGF receptor is a major transducing element in this pathway. Interestingly, tyrphostin AG1478 did not affect integrin-induced MAP kinase activation in parental NIH-3T3 cells (see Figure 6), indicating that an EGF receptor-independent pathway, most likely involving Shc–caveolin complexes as described by Wary *et al.* (1996), is utilized in these cells to regulate MAP kinase activation in response to extracellular matrix. Since parental NIH-3T3 cells express very low levels of EGF receptors (~5×10³) compared

with endothelial cells, human fibroblasts and EGFR-transfected NIH-3T3 cells (>3×10⁴ molecules per cell), we propose that integrins utilize the EGF receptor as a transducing molecule to activate MAP kinases when this receptor is expressed above a threshold level.

The level of EGF receptor phosphorylation induced by integrins can be increased strongly by addition of EGF (see Figure 1D), indicating that integrins induce only partial activation of the EGF receptor. The partial activation of the receptor is also confirmed by the fact that Erk-1/MAP kinase activation induced by integrin-mediated adhesion is lower than that obtained in response to EGF in adherent cells. The level of Erk-1/MAP kinase activation induced by the integrin-dependent EGF receptor pathway is not sufficient to promote cell growth, consistent with the finding that G_1-arrested EGFR-transfected NIH-3T3 cells do not progress into the cell cycle when plated on fibronectin in the absence of growth factors. Proliferation was observed in cells exposed to fibronectin in the presence of EGF, suggesting that the increased level of MAP kinase activation obtained by co-stimulation with matrix proteins and EGF is necessary to induce cell proliferation (see Table I). The requirement for signaling originating from cell adhesion and from growth factor stimulation to induce MAP kinase activation has also been reported by other investigators (Zhu and Assoian, 1995; Lin *et al.*, 1997; Renshaw *et al.*, 1997). Therefore, we propose a model in which a threshold level of growth factor receptor activation induced by cell–matrix interaction is required in order to obtain the full response of the receptor to its ligand. Other investigators have shown that EGF stimulates EGF receptor tyrosine phosphorylation and MAP kinase induc-

tion more efficiently when integrins are occupied (Cybulsky *et al.*, 1994; Miyamoto *et al.*, 1996; Cybulsky and McTavish, 1997; Jones *et al.*, 1997). These authors did not detect in their systems the integrin-dependent EGF receptor activation which we report here, most probably due to the different experimental protocols used. In fact, their cells were always maintained in the presence of EGF or serum, a possible source of TGF-α. The ability of integrins to transactivate EGF receptor, as reported in our work, can thus represent a molecular mechanism at the basis of this phenomenon. Assuming that integrins can transactivate growth factor receptors other than the EGF receptor, the data presented here can also explain the ability of integrins to potentiate signaling pathways in response to insulin and PDGF, as shown by Vuori and Ruoslahti (1994) and Schneller *et al.* (1997). These authors reported that upon growth factor stimulation, both insulin and PDGF-β receptors are highly tyrosine phosphorylated and bind to several signaling molecules, such as IRS-1, PLCγ, Ras GAP, the p85 subunit of PtdIns-3 kinase and the tyrosine phosphatase SHP2, when αvβ3 integrin is occupied by its matrix ligand. In endothelial cells, moreover, we have shown that αvβ3 integrin can potentiate the activation of vascular endothelial growth factor receptor by its ligand (R.Soldi, S.Mitola, M.Strasly, P.Defilippi, G.Tarone and F.Bussolino, submitted). Indeed, adhesion-dependent growth factor receptor activation is not restricted to the EGF receptor. It has been shown, in fact, that cell–substratum interactions stimulate phosphorylation of the hepatocyte growth factor (HGF) receptor (Rusciano *et al.*, 1996; Wang *et al.*, 1996) and PDGFβ receptor (Sundberg and Rubin, 1996). Moreover, cell–cell interactions mediated by the neural cell adhesion molecule activate the fibroblast growth factor receptor in neuronal cells (Saffell *et al.*, 1997), suggesting that activation of growth factor receptors in the absence of their specific ligands can be a broadly used mechanism in adhesion-mediated signaling and represents a priming step in order to obtain a full response to growth factor.

The molecular mechanisms underlying EGF receptor activation by integrins remain to be defined. Our findings indicate that integrin-dependent activation of the EGF receptor and of other tyrosine kinases such as p125Fak occurs through distinct mechanisms. In fact, cytochalasin D, which disrupts the actin cytoskeleton, strongly inhibits p125Fak tyrosine phosphorylation, but does not affect EGF receptor tyrosine phosphorylation in response to integrins. This indicates that the organization of actin cytoskeleton is not a primary event in integrin-dependent EGF receptor activation, while it is required to bring together signaling proteins leading to p125Fak tyrosine phosphorylation. These data also indicate that p125Fak tyrosine phosphorylation is not required in integrin-mediated EGF receptor activation. Moreover, integrins and EGF receptors can associate on the membrane, forming a molecular complex, as shown by co-immunoprecipitation experiments, while association of integrins with p125Fak cannot be detected by this technique. Preliminary experiments also show that PtdIns-3 kinase is not involved in integrin-induced EGF receptor tyrosine phosphorylation, as detected by the use of specific inhibitors (M.Venturino, L.Dolce and P.Defilippi, unpublished results).

In addition to inducing Erk-1/Map kinase activity, integrin-mediated EGF receptor activation can also generate downstream signaling leading to anchorage-dependent cell survival. It is well known that cells undergo apoptosis when they lose contact with the extracellular matrix (reviewed in Frisch and Ruoslahti, 1997). Integrin-induced tyrosine phosphorylation may indeed represent one signaling mechanism to regulate cell survival in response to adhesion. Indeed, inhibition of tyrosine phosphatases prevents apoptosis in cells in suspension (Meredith *et al.*, 1993). Microinjection of p125Fak antibodies (Hungerford *et al.*, 1996) induces apoptosis in embryo fibroblasts, while expression of a constitutive active form of this kinase rescues epithelial cells from apoptosis (Frisch *et al.*, 1996), indicating that p125Fak is involved in survival signaling. The data reported here show that in cells expressing EGF receptor, treatment with tyrphostin AG1478 strongly reduces survival of cells plated on matrix in the absence of growth factors. Similar results are observed in cells expressing the dominant-negative form of the EGF receptor. The downstream pathway involved is likely to be independent of Erk-1/MAP kinase activation, since the specific inhibitor of the MAP kinase kinase MEK, PD 98059 (Dudley *et al.*, 1995), does not interfere with adhesion-induced cell survival. PtdIns-3 kinase has been implicated recently as a key mediator of matrix-induced survival of normal epithelial cells (Khwaja *et al.*, 1997). Interestingly, in cells expressing EGF receptor, fibronectin-mediated cell survival is blocked by the PtdIns-3 kinase inhibitor wortmannin, suggesting that PtdIns-3 kinase may be involved in the downstream pathways leading to cell survival. Therefore, integrin-mediated EGF receptor activation constitutes a novel signaling pathway utilized by integrins to promote adhesion-dependent cell survival.

Here we show that the EGF receptor can be activated by interaction with extracellular matrix receptors. The EGF receptor recently has been shown to provide a link to MAPK activation in response to G protein-coupled receptor agonists (Daub *et al.*, 1996, 1997; Luttrell *et al.*, 1997) and to growth hormone receptor, which belongs to the cytokine receptor superfamily (Yamauchi *et al.*, 1997), and to modulate ion channel activity following m1 muscarinic acetylcholine receptor activation (Tsai *et al.*, 1997). In addition, a variety of stimuli, including UV irradiation or calcium-dependent responses, result in ligand-independent EGF receptor transactivation (Huang *et al.*, 1996; Rosen and Greenberg, 1996), suggesting that the EGF receptor may represent a switch point for multiple stimuli regulating a variety of fundamental physiological processes.

All together, our results demonstrate that the EGF receptor can act as a downstream effector in integrin signaling upon cell–matrix interactions. EGF receptor activation by integrins is partial, compared with that obtained with EGF. Nevertheless, this activation is sufficient to prevent apoptosis induced by cell–matrix detachment, and this event may represent a prerequisite for a full mitogenic response to growth factor stimulation in adherent compared with non-adherent cells. In conclusion, the ability of integrins to activate the EGF receptor and downstream signaling further supports the concept that integrins and growth factor receptors can cooperate directly in the plane of the membrane to trigger specific signaling.

Materials and methods

Reagents and antibodies

Fibronectin and vitronectin were purified from human plasma as previously described (Defilippi *et al.*, 1994). Collagen type I, poly-L-lysine, cycloheximide, cytochalasin D, myeline basic protein (MBP), bovine pancreatic RNase, propidium iodide, puromicin, wortmannin, sulfosuccinimidyl biotin and human recombinant EGF were all from Sigma Chemical Co. Tyrphostin AG1478 and PD98059 were from Calbiochem. Protein A–Sepharose was from Pharmacia. [γ-^{32}P]ATP, nitrocellulose, streptavidin–horseradish peroxidase (HRP) conjugate, the ECL reagents and films were from Amersham. Culture media, sera and the Lipofectamine reagent were from Gibco-BRL.

The following antibodies to integrin subunits were used: monoclonal antibody (mAb) BV7 to the human β1 integrin subunit (purchased from Bioline Diagnostici), mAb L230 to the αv integrin subunit purchased from ATTC, mAb B212 to the β3 subunit and the polyclonal antibody to the β1 integrin cytoplasmic domain previously described (Defilippi *et al.*, 1991). mAb RI.30 to human β2-microglobulin was a kind gift of Professor F.Malavasi (University of Ancona, Italy). All the monoclonal antibodies were affinity purified on protein A–Sepharose as described (Ey *et al.*, 1978), and the purity of the antibodies was >95%. Antibodies to the EGF receptor were: mAb HB-8505 (purchased from ATCC), polyclonal antibody 2913 (Beguinot *et al.*, 1986) and polyclonal antibody EGFR1, produced by injection of a 13 amino acid peptide corresponding to the C-terminus of the human EGF receptor (NH$_2$-LRVAPQSSEFIGA-COOH). Polyclonal antibody to p125Fak Fak4 has been described previously (Defilippi *et al.*, 1995). Polyclonal antibody to Shc was produced by injection of a GST fusion protein corresponding to amino acid residues 1180–1620 of human Shc (Pellicci *et al.*, 1992). Rabbit anti-mouse IgG was produced and purified in our laboratory. mAb PY20 and RC20 to phosphotyrosine (anti-PY) were from Transduction Laboratories. Polyclonal antibody C-16 to Erk-1/MAP kinase was from Santa Cruz Biotechnology.

Cell culture and transfection

Human dermal fibroblasts were prepared from skin biopses of a healthy donor and stabilized in culture according to established procedures. Human endothelial cell line ECV304 was a kind gift of Dr R.Pardi (Dibit, Milano). NIH-3T3 cells tranfected with wild-type human EGF receptor (EGFR NIH-3T3) (Velu *et al.*, 1989) or EGF receptor kinase-negative point mutants (EGFR/K⁻) have been described previously (Sorkin *et al.*, 1992; Alvarez *et al.*, 1995). The dominant-negative EGF receptor mutant lacking all the kinase and C-terminal domains (EGFR/ΔC) was prepared by PCR mutagenesis by inserting point mutations in EGF receptor cDNA in positions 2248 and 2257, converting Glu633 and Glu666 into stop codons. PCRs were performed in the pMMTV-EGFR as previously described (Sorkin *et al.*, 1996) using an upstream primer 5'-TACGCAGACGCCGGCCATGT3' (2014–2036, *Nae*I) and downstream primer 5'-GACCATGATCATGTA-GACATCGATGGGT-3' (3033–2994, *Cla*I). For mutagenesis, primers 5'-CTG-CAGGAGAGGTAGCTTGTGTAGCCTTACA-3' (226–2268, direct) and 5'-TGTAAGGAGGCTACACACAAGATCCCTCCCTGCAG-3' (2268–2236, inverse) were used. The *Nae*I–*Cla*I-mutated fragment was recloned back into the pMMTV-EGFR and sequenced to confirm the mutations and ensure the absence of secondary mutation. The *Not*I–*Eco*RV fragment of EGFR/ΔC cDNA was subcloned into the pRK-5 vector (pRK-EGFR/ΔC). NIH-3T3 cells expressing wild-type EGF receptor were transfected by the Lipofectamine method with pRK-EGFR/ΔC together with the pGKpuro plasmid encoding the puromycin-resistant gene, and cells were selected with 3.5 μg/ml puromicin. To analyze expression of the EGFR/ΔC as a protein migrating at 130 kDa, cells were cell surface biotinylated as described previously (Defilippi *et al.*, 1997b), and EGF receptor was immunoprecipitated with mAb HB-8509 and revealed with streptavidin–HRP (Amersham).

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (N6 or ECV304) or newborn calf serum (NIH-3T3 cells).

Adhesion assay and integrin clustering

Cells grown to confluence were serum deprived in DMEM for 16–24 h, pre-treated for 2 h with 20 μM cycloheximide, detached with 10 mM EDTA in phosphate-buffered saline (PBS), washed and kept in suspension or plated for the indicated times on poly-L-lysine, matrix proteins or integrin antibody-coated dishes as previously described (Defilippi *et al.*, 1994). When indicated, human recombinant EGF, tyrphostin AG1478

or cytochalasin D were added. In the co-immunoprecipitation experiments, cell surface biotinylation was performed as previously described (Defilippi *et al.*, 1997b). Cells were then washed with a PBS buffer containing 5 mM EDTA, 10 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 0.4 mM Na$_3$VO$_4$ and detergent extracted in lysis buffer as described below. Integrin clustering was performed as previously described (Defilippi *et al.*, 1994).

Cell lysis, immunoprecipitation and immunoblotting

Cells were extracted with 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–HCl pH 8, 5 mM EDTA, 10 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 0.4 mM Na$_3$VO$_4$, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 U/ml aprotinin). Cell lysates were centrifuged at 13 000 *g* for 10 min, and the supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method (Bio-Rad). Aliquots of 100 μg of proteins were run on SDS–PAGE under reducing conditions. For immunoprecipitation experiments, 1–3 mg of proteins were immunoprecipitated with the appropriate antibody for 1 h at 4°C as previously described (Defilippi *et al.*, 1991) in the presence of 50 μl of protein A–Sepharose beads. Following SDS–PAGE, proteins were transferred to nitrocellulose, reacted with specific antibodies and then detected with HRP-conjugated secondary antibodies and chemiluminescent ECL detection. When appropriate, the nitrocellulose membranes were stripped according to the manufacturers' recommendations and reprobed.

EGF receptor kinase assay

Cells were extracted in buffer containing 15% glycerol, 150 mM NaCl, 50 mM Tris–HCl pH 8, 5 mM EDTA, 10 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 1 mM Na$_3$VO$_4$, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 U/ml aprotinin, immunoprecipitated with EGF receptor polyclonal antibodies, and washed and incubated in EGFR kinase buffer [20 mM PIPES pH 7.2, 10 mM MnCl$_2$, 1 mM dithiothreitol (DTT), 100 μM Na$_3$VO$_4$, 10 μM ATP] in the presence of 2 μCi of [γ-^{32}P]ATP at 30°C for 3 min. To measure EGF receptor kinase activity on exogenous substrate, 5 μg of MBP for each sample were added to the reaction mixture. Reactions were terminated by addition of 2× Laemmli buffer and subjected to SDS–PAGE. γ-^{32}P-Labeled EGF receptor was detected by autoradiography and quantified by densitometric analysis using the GS 250 Molecular Imager (Bio-Rad). EGF receptor was quantified by Western blotting with polyclonal antibody EGFR1.

Erk-1 MAPK assay

Erk-1 was immunoprecipitated from cell extracts prepared with 1% NP-40 lysis buffer using 2 μg of polyclonal Erk-1 antibody C-16. Immunoprecipitates additionally were washed with MAPK buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$) and incubated in the presence of a 5 μg/sample of MBP and 2 μCi of [γ-^{32}P]ATP in the same buffer at 30°C for 20 min. Reactions were stopped by addition of 2× Laemmli buffer and subjected to SDS–PAGE. γ-^{32}P-Labeled MBP was detected by autoradiography and quantified using the GS 250 Molecular Imager (Bio-Rad).

Analysis of cell cycle progression and apoptosis

To monitor progression to G$_2$/M phases of the cell cycle, EGFR-transfected NIH-3T3 cells were synchronized by 30 h serum deprivation, detached with 0.05% trypsin, 0.02% EDTA and kept in suspension or plated for 24 h on 10 μg/ml fibronectin-coated dishes in the absence or presence of 10% FCS or 50 ng/ml of human recombinant EGF. Cells were then detached with trypsin/EDTA, fixed in 75% ethanol for 2 min at room temperature and centrifuged. The cell pellet was resuspended in PBS containing 1% NP-40, 1 mg/ml bovine pancreatic RNase and 30 μg/ml propidium iodide for 1 h at room temperature in the dark. Propidium iodide fluorescence was analyzed on the flow cytometer Bryte HS (Bio-Rad), and cell cycle analysis was performed according to the Bio-Rad DNA-Analysis software.

To detect apoptosis, cells were kept in suspension on polyHEMA-coated dishes or plated on fibronectin-coated dishes in serum-free medium in the presence of different mediators for 24 h as previously described (Bozzo *et al.*, 1997). Cells were then stained with 25 μg/ml propidium iodide and examined in a Bio-Rad 600 confocal microscope using the argon laser (Bio-Rad Microscience Division, Hercules, CA). Chromatin condensation and nuclear fragmentation were used as morphological criteria to quantify apoptotic cells.

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Stimulation of growth factor receptor signal transduction by activation of voltage-sensitive calcium channels

(epidermal growth factor receptor/Ras/mitogen-activated protein kinase)

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ABSTRACT   To understand the mechanisms by which electrical activity may generate long-term responses in the nervous system, we examined how activation of voltage-sensitive calcium channels (VSCCs) can stimulate the Ras/mitogen-activated protein kinase (MAPK) signaling pathway. Calcium influx through L-type VSCCs leads to tyrosine phosphorylation of the adaptor protein Shc and its association with the adaptor protein Grb2, which is bound to the guanine nucleotide exchange factor Sos1. In response to calcium influx, Shc, Grb2, and Sos1 inducibly associate with a 180-kDa tyrosine-phosphorylated protein, which was determined to be the epidermal growth factor receptor (EGFR). Calcium influx induces tyrosine phosphorylation of the EGFR to levels that can activate the MAPK signaling pathway. Thus, ion channel activation stimulates growth factor receptor signal transduction.

Calcium influx into neurons is the critical transducer of electrical input into biochemical output (1). A wide range of neurotransmitter receptors and second messenger systems have been shown to regulate the influx of extracellular calcium through their effects on voltage-sensitive calcium channels (VSCCs) and other ion channels in a process termed neuromodulation (2–4). However, the mechanisms by which calcium influx elicits long-term neuronal responses are less clear.

Studies of the biochemical responses generated in response to calcium influx have focused on activation of cytoplasmic signaling molecules that directly bind calcium or calcium–calmodulin (CaM) complexes, such as calcium–CaM-dependent adenylate cyclases and protein kinases (5). Calcium influx also leads by indirect mechanisms to activation of the ubiquitous mitogen-activated protein kinase (MAPK) pathway (6, 7), which is a critical intermediate in long-term cellular responses such as proliferation and differentiation (8–10). Recently, we have demonstrated that stimulation of the MAPK pathway in response to calcium influx through L-type VSCCs involves activation of the small guanine nucleotide binding protein Ras (11), a protooncogene product that mediates MAPK activation in response to a wide variety of mitogens, cytokines, and trophic factors such as nerve growth factor (NGF) (8–10). To understand the mechanisms by which electrical activity may initiate long-term responses in the nervous system, we examined how calcium influx leads to activation of Ras.

Growth factors initiate signaling processes that lead to Ras activation by binding to transmembrane receptors that contain intrinsic tyrosine kinase activity or, in the case of cytokine receptors, that are associated through their cytoplasmic domains with nontransmembrane protein tyrosine kinases such as the Src family members (12–14). Ligand binding induces receptor dimerization and autophosphorylation on tyrosine residues. These phosphorylated tyrosines create binding sites for Src homology 2 (SH2) domains, which are present in a number of different signaling molecules that associate with activated growth factor receptors (15, 16). SH2 domains bind to phosphorylated tyrosine residues and adjacent amino acid sequences, which determine the specificity of the interaction.

One class of signaling molecule that inducibly binds to growth factor receptors is the adaptor protein, which lacks catalytic moieties but mediates protein–protein interactions via modular domains such as SH2 domains. One of the adaptor proteins that inducibly associates with tyrosine-phosphorylated growth factor and cytokine receptors is the SH2/collagen protein (Shc) (17). Shc is itself also inducibly phosphorylated on tyrosine in response to growth factor and cytokine stimulation (18–22), which creates a consensus binding site (pYXN) that is recognized by another SH2 domain-containing adaptor protein, growth factor receptor binding protein 2 (Grb2) (23). Grb2 contains, in addition to its SH2 domain, two SH3 domains that mediate its interaction with proline-rich sequences in the Ras guanine nucleotide exchange factor (GEF) termed mSos1 (24–28). Induction of Grb2–Sos1 association with Shc through Y317 is a potential mechanism for Ras activation in response to growth factors and cytokine stimulation (29–35).

The parallels we found previously between calcium and growth factor activation of MAPK suggested that tyrosine phosphorylation might be involved in calcium activation of Ras. We report here that calcium influx upon activation of VSCCs leads to tyrosine phosphorylation of Shc and its association with Grb2 and Sos1. In addition, this signaling complex inducibly associates with the epidermal growth factor receptor (EGFR), which is phosphorylated on tyrosine in response to calcium influx to a level that is sufficient to lead to downstream MAPK activation. Our results demonstrate that growth factor receptor signal transduction is activated in response to VSCC stimulation, which may be an important biochemical mechanism by which neuronal activity can generate long-term cellular responses.

MATERIALS AND METHODS

Materials. EGF was from Collaborative Biomedical Products (Bedford, MA), and NGF was purified from mouse salivary glands as described (36). Nifedipine was from Sigma. Anti-Trk antibodies were the generous gift of David Kaplan (67). Other antibodies were obtained from the following vendors: anti-phosphotyrosine [Tyr(p)] monoclonal antibody (mAb) 4G10 from Upstate Biotechnology (Lake Placid, NY); anti-Tyr(p) mAb PY20 from ICN; anti-Shc polyclonal antibody (pAb) and mAb from Transduction Laboratories (Lexington, KY); anti-Grb2 mAb from Upstate Biotechnology; anti-Grb2

Abbreviations: VSCC, voltage-sensitive calcium channel; CaM, calmodulin; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; GEF, guanine nucleotide exchange factor; EGF, epidermal growth factor; EGFR, EGF receptor; mAb, monoclonal antibody; pAb, polyclonal antibody.
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pAb from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Sos1 pAb from Upstate Biotechnology; anti-EGFR pAb from Upstate Biotechnology; anti-ErbB2 from Oncogene Science; goat anti-mouse pAb from Calbiochem; and rabbit anti-sheep pAb from Pierce.

Cell Culture and Stimulation. PC12 cells were obtained from Simon Halegoua (68) and cultured on 100-mm tissue culture dishes (Falcon) in DMEM (GIBCO) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (GIBCO) in a humidified incubator with 10% CO_2/90% air. Cell membranes were depolarized by addition of an isosmotic solution of KCl (or NaCl control) to a final concentration of 50 mM as described (11).

Immunoprecipitation and Immunoblotting. Cells were lysed in HNTG buffer [50 mM Hepes, pH 7.5/50 mM NaCl/1% Triton X-100/10% glycerol (vol/vol)/1.5 mM $MgCl_2$/1 mM EDTA/10 mM sodium pyrophosphate/1 mM Na_3VO_4/100 mM NaF/30 mM 2-(p-nitrophenyl) phosphate/1 mM phenyl-methylsulfonyl fluoride/10 μg of aprotinin per ml/10 μg of leupeptin per ml] and centrifuged at 10,000 × g for 15 min. Supernatants were mixed with primary antibody and rocked at 4°C for 1–4 h. Secondary antibody and protein A-Sepharose (Calbiochem) were added for an additional 1–2 h. Immunoprecipitates were washed three times in HNTG and resuspended in 2× Laemmli sample buffer (80 mM Tris·HCl, pH 6.8/15% glycerol/2% SDS/0.01% bromphenol blue/10% 2-mercaptoethanol). Precipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose, and analyzed by Western blotting as described (11). Antibody binding was detected by enhanced chemiluminescence (ECL; Amersham) with a secondary antibody conjugated to horseradish peroxidase. For analysis of Shc, Grb2, and Sos1 coimmunoprecipitation, 6–12% gradient gels were run, and the nitrocellulose blots were cut horizontally at the 125- and 35-kDa markers for blotting of individual proteins.

RESULTS

To examine whether tyrosine phosphorylation might be involved in calcium activation of Ras, we determined the effect of membrane depolarization on tyrosine phosphorylation of Shc and its association with Grb2 in the pheochromocytoma cell line PC12. Membrane depolarization of both NGF-differentiated and undifferentiated PC12 cells has been demonstrated to activate signaling pathways that are also activated by direct electrical stimulation of primary neurons in culture as well as by neuronal excitation *in vivo* (37). Undifferentiated PC12 cells were membrane depolarized by exposure to elevated levels of extracellular KCl to induce calcium influx through VSCCs, or cells were treated with NGF or EGF. Shc proteins were immunoprecipitated and analyzed by protein immunoblotting with antibodies to phosphotyrosine. Equal amounts of Shc were immunoprecipitated as shown by immunoblotting for Shc proteins (Fig. 1A *Bottom*). KCl induced tyrosine phosphorylation of the 48- and 56-kDa Shc isoforms, as did NGF and EGF treatment (Fig. 1A *Top*). The 65-kDa Shc isoform was inducibly tyrosine phosphorylated to a lesser extent by NGF and EGF but not by KCl. Although this may simply be due to a detection limit in the assay, the differential phosphorylation of the three Shc isoforms in response to the different stimuli may reflect specificity in the signaling pathways. These results demonstrate that membrane depolarization leads to inducible tyrosine phosphorylation of Shc, an adaptor protein that is involved in Ras activation in response to growth factor stimulation.

To determine whether KCl-induced Shc phosphorylation led to its functional association with Grb2, Shc immunoprecipitates (Fig. 1A) from PC12 cells treated with KCl, NGF, or EGF were analyzed for coprecipitation of Grb2 by immunoblotting with anti-Grb2 antibody. Membrane depolarization
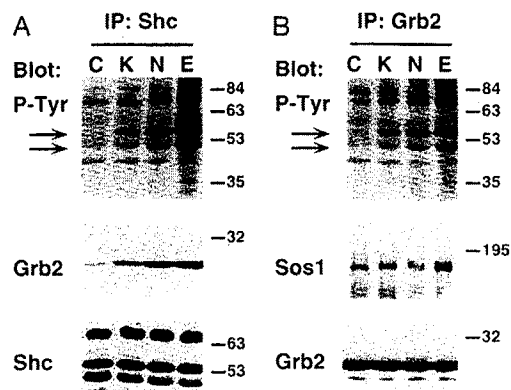

Fig. 1. Calcium induction of Shc tyrosine phosphorylation and Grb2 association. (*A*) PC12 cells were incubated with 50 mM NaCl control solution (lane C), 50 mM KCl (lane K), 100 ng of NGF per ml (lane N), or 10 ng of EGF per ml (lane E) for 5 min. Lysates were immunoprecipitated with anti-Shc pAb. Washed immunoprecipitates (IP) were separated by PAGE and transferred to nitrocellulose for immunoblotting with a mixture of anti-Tyr(p) antibodies 4G10 and PY20, anti-Grb2 mAb (*Middle*), or anti-Shc mAb (*Bottom*). Positions of migration of prestained molecular size markers (kDa) (Sigma) are shown. Arrows indicate phosphorylated Shc isoforms of 48 and 56 kDa. (*B*) PC12 cells were treated as in *A*. Lysates were immunoprecipitated with a pAb against Grb2, and precipitated proteins were analyzed by immunoblotting with anti-Tyr(p) antibodies (*Top*), anti-Sos1 pAb (Upstate Biotechnology; *Middle*), or anti-Grb2 mAb (*Bottom*) to confirm that equal levels of Grb2 were immunoprecipitated. Positions of migration of prestained molecular size markers are shown.

with KCl led to the inducible association of Shc with Grb2, as did NGF and EGF treatment (Fig. 1A *Middle*). This KCl-induced association of Shc with Grb2 was also demonstrated by first immunoprecipitating Grb2 and then immunoblotting with antibodies to phosphotyrosine to detect coprecipitated Shc proteins (Fig. 1B *Top*). In addition, by immunoblotting Grb2 immunoprecipitates with antibodies to Sos1, we found that Grb2 is constitutively bound to Sos1 in PC12 cells, as others have previously shown (Fig. 1B *Middle*) (38). Thus, KCl-stimulated tyrosine phosphorylation of Shc can induce its association with the Grb2–Sos1 complex.

Evidence suggests that the Ras GEF must be targeted to the plasma membrane in order to activate Ras (39). In the case of growth factor stimulation, this can be accomplished by association of the adaptor protein–GEF complexes with the receptor tyrosine kinase itself. This can occur by receptor binding directly to Grb2–Sos1 complexes through the Grb2 SH2 domain (24–28) or through binding Shc–Grb2–Sos1 complexes through the Shc SH2 domain (33–35, 40–42) or a phosphotyrosine-binding domain at the Shc N terminus (43, 44). To determine how calcium influx might target the Shc–Grb2–Sos1 signaling complex to the plasma membrane, we examined whether KCl treatment led to association of Shc, Grb2, or Sos1 with a tyrosine-phosphorylated protein that could act as a membrane anchor. Shc, Grb2, and Sos1 proteins were immunoprecipitated and analyzed for coprecipitating proteins containing phosphotyrosine by immunoblotting with anti-phosphotyrosine antibodies. KCl induced the association of an ≈180-kDa tyrosine-phosphorylated protein with Shc, Grb2, and Sos1 (Fig. 2). Surprisingly, this protein comigrated with a tyrosine-phosphorylated protein that was coprecipitated in response to EGF stimulation. A low or undetectable level of pp180 was detected in Shc, Grb2, and Sos1 immunoprecipitates from NGF-treated cells. We failed to detect coprecipitation of the NGF receptor p140^{trk} in Shc immunoprecipitates in response to NGF, possibly because of lower endogenous levels of Trk than EGFR in these cells.

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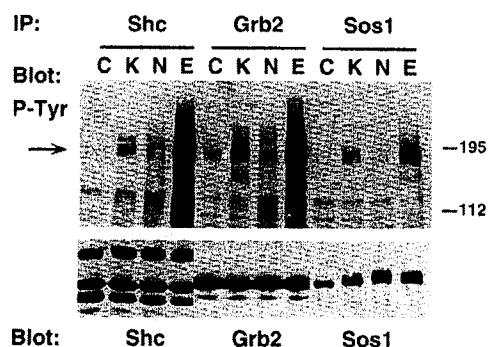*Proc. Natl. Acad. Sci. USA 93 (1996)*   1115

FIG. 2. Calcium-induced association of Shc, Grb2, and Sos1 with pp180. PC12 cells were treated with 50 mM NaCl control solution (lanes C), 50 mM KCl (lanes K), 100 ng of NGF per ml (lanes N), or 10 ng of EGF per ml (lanes E) for 5 min. Lysates were immunoprecipitated with pAb against Shc, Grb2, or Sos1, and precipitated proteins (IP) were analyzed by immunoblotting with anti-Tyr(p) antibodies (*Top*) or with antibodies to the precipitated proteins to confirm equal recovery (*Bottom*). Positions of migration of prestained molecular size markers (kDa) are shown. Arrow indicates pp180.

The EGFR can associate with Grb2 directly through binding of the Grb2 SH2 domain to phosphorylated Y1068 or Y1086 in the receptor, as well as indirectly via binding of Shc to phosphorylated Y1148 or Y1173 (41, 42, 45). The observation that a 180-kDa protein inducibly associated with Shc, Grb2, and Sos1 in response to KCl as well as EGF raised the possibility that both proteins might be the EGFR. This possibility was examined directly by immunoprecipitating Grb2 and immunoblotting with antibodies to the EGFR. Membrane depolarization with KCl induced Grb2 association with the EGFR, although to a lesser extent than treatment with 10 ng of EGF per ml (Fig. 3, lanes 1–3). This result suggests that the 180-kDa protein that is coprecipitated with Shc, Grb2, and Sos1 in response to KCl stimulation is the EGFR. Thus, calcium influx induces the association of a growth factor receptor with downstream signaling proteins that can trigger Ras activation.

To determine whether KCl-induced EGFR association with Shc, Grb2, and Sos1 was due to calcium influx through VSCCs, the effect of a specific channel antagonist on the interaction was examined. Pretreatment of PC12 cells with the L-type VSCC antagonist nifedipine or with the calcium chelator EGTA for 15 or 5 min, respectively, completely blocked the ability of KCl to induce the association of Grb2 with the

EGFR, whereas they had no effect on EGF-induced association (Fig. 3, lanes 4–9). These results demonstrate that KCl-induced association of the EGFR with Grb2 is dependent on the influx of extracellular calcium through L-type VSCCs and is not a nonspecific effect of membrane depolarization. This finding is consistent with previous observations that nifedipine and nimodipine, another dihydropyridine antagonist of L-type VSCCs, block KCl activation of Ras and that KCl activation of MAPK is blocked by nifedipine but not by ω-conotoxin, an inhibitor of N-type VSCCs (ref. 11; unpublished data). The importance of L-type VSCCs in mediating the signaling effects of membrane depolarization we observe reflects the fact that they are the primary carrier of voltage-sensitive calcium current in undifferentiated PC12 cells (46).

Since Shc and Grb2 specifically recognize tyrosine-phosphorylated proteins, the finding that these adaptor molecules inducibly associate with the EGFR upon VSCC activation suggested that calcium influx was leading to tyrosine phosphorylation of the receptor. To examine the effect of calcium influx on the phosphotyrosine content of the EGFR, the EGFR was immunoprecipitated after KCl or EGF treatment and analyzed by immunoblotting with antibodies to phosphotyrosine. Calcium influx led to inducible tyrosine phosphorylation of the EGFR within 20 sec of membrane depolarization (Fig. 4 *Top*). This calcium-induced phosphorylation of the EGFR can therefore account for the inducible association of the receptor with Shc–Grb2–Sos1 in response to membrane depolarization.

To determine whether calcium-induced EGFR tyrosine phosphorylation was likely to lead to physiologically meaningful receptor responses, we titrated down the dose of EGF to a level (1 ng/ml) that produced an induction of EGFR tyrosine phosphorylation comparable to that induced by KCl (Fig. 4 *Top*). We then examined whether this level of EGFR phosphorylation was sufficient to produce downstream responses to EGF. MAPK activation was monitored as an indicator of physiologically important signal transduction, since MAPK activation has been demonstrated to be critical for a variety of long-term cellular responses to extracellular stimuli, including cell proliferation and differentiation (8–10). Both membrane depolarization and treatment with EGF (1 ng/ml) led to inducible tyrosine phosphorylation of the 42- and 44-kDa isoforms of MAPK. The identification of these tyrosine phosphorylated bands as activated MAPK was confirmed by immunoblotting with antibodies that specifically recognize the
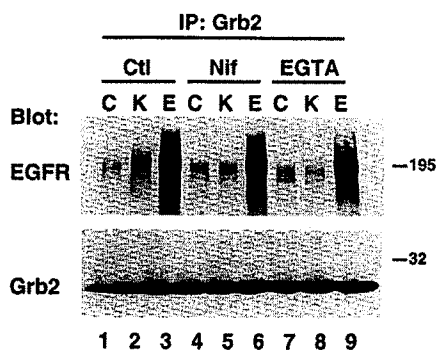

FIG. 3. Calcium induction of Grb2 association with the EGFR. PC12 cells were treated with 50 mM NaCl control solution (lanes C), 50 mM KCl (lanes K), or 10 ng of EGF per ml (lanes E) for 2 min. Lysates were immunoprecipitated with anti-Grb2 pAb, and precipitated proteins (IP) were analyzed by immunoblotting with anti-EGFR pAb (*Top*) or anti-Grb2 mAb (*Bottom*). Cells were pretreated as follows: lanes 1–3, vehicle control for 15 min (Ctl); lanes 4–6, 5 μM nifedipine for 15 min (Nif); lanes 7–9, 3 mM EGTA for 5 min (EGTA). Positions of migration of prestained molecular size markers (kDa) are shown.

Blots: P-Tyr

FIG. 4. Calcium induction of EGFR tyrosine phosphorylation and downstream signaling. PC12 cells were treated with 50 mM KCl or 1 ng of EGF per ml for the indicated times. Lysates were immunoprecipitated with anti-EGFR pAb, and immunoprecipitated proteins (IP) (*Top*) or samples of lysates (*Bottom*) were analyzed by immunoblotting with anti-Tyr(p) antibodies. Positions of migration of prestained molecular size markers (kDa) are shown. Arrows indicate positions of the 42- and 44-kDa MAPK isoforms.

phosphorylated and activated form of MAPK (L.B.R., David D. Ginty, and M.E.G., unpublished observations). Thus, calcium influx in response to L-type VSCC activation leads to tyrosine phosphorylation of the EGFR to an extent that is sufficient to induce downstream signaling to MAPK. Notably, the dose of EGF used in this experiment is 10-fold higher than doses reported to generate physiological responses in neurons (47, 48), suggesting that the comparable level of EGFR tyrosine phosphorylation produced by calcium influx is likely to be a physiologically important mechanism of signal transduction in response to neuronal activity.

Finally, we addressed the generality and specificity of the calcium signaling response by examining whether KCl induced tyrosine phosphorylation of other growth factor receptors. KCl treatment led to tyrosine phosphorylation of the EGFR family member ErbB2 (Fig. 5A) as well as a 100-kDa protein that is recognized by an antibody generated against a consensus tyrosine kinase domain (unpublished data). In contrast, KCl did not induce tyrosine phosphorylation of the insulin receptor (unpublished data) or the NGF receptor Trk, which was robustly phosphorylated in response to NGF (Fig. 5B). These results suggest that calcium influx may activate the signal transduction pathways of other receptor tyrosine kinases in addition to the EGFR but that there is specificity in the signaling responses generated. Thus, stimulation of growth factor receptor signaling may be a general mechanism by which calcium influx generates long-term responses in cells.

DISCUSSION

Our results demonstrate that activation of VSCCs can lead to tyrosine phosphorylation of the EGFR and its association with the adaptor proteins Shc and Grb2 and the guanine nucleotide exchange factor Sos1. A number of studies have demonstrated that Shc, Grb2, and Sos1 act as signaling mediators in growth factor receptor activation of Ras. Taken together, these findings provide a mechanism by which calcium influx could activate the Ras/MAPK pathway and demonstrate that growth factor receptors can be functionally coupled to their downstream signaling pathways in response to ion-channel activation.

The mechanism by which calcium influx induces EGFR tyrosine phosphorylation is not yet clear. Although it is possible that L-type VSCC activation could lead to the calcium-induced release of EGF and autocrine stimulation of the receptor, we favor a model of ligand-independent EGFR activation by calcium for a number of reasons. The EGFR precursor is a transmembrane protein not known to be packaged in secretory vesicles (49), and we have found that a neutralizing antibody to EGF prevents tyrosine phosphoryla-

tion of the EGFR in response to EGF but not in response to L-type VSCC activation (unpublished data). Thus, calcium does not lead to EGFR tyrosine phosphorylation by autocrine release of EGF itself. We think a more likely mechanism could involve calcium activation of a cytoplasmic tyrosine kinase that could phosphorylate the receptor C-terminal tail (Fig. 6). This trans-phosphorylation of the EGFR by a calcium-responsive cytoplasmic tyrosine kinase may then activate the EGFR kinase so that it autophosphorylates on the same sites that are phosphorylated in response to EGF binding. Members of the Src family of cytoplasmic tyrosine kinases are good candidates for mediating calcium-induced tyrosine phosphorylation of the EGFR. Although we are unaware of evidence that Src directly binds the EGFR, Src transformation of fibroblasts leads to tyrosine phosphorylation of the EGFR and likely to activation of its kinase activity as well (50). In addition, Src is activated in response to ionomycin treatment of keratinocytes (51), and targeted gene disruption of the Src family member fyn suggests that the Fyn protein may play a role in calcium-dependent responses in the nervous system, such as synaptic potentiation and memory formation (52). Alternatively, calcium influx could increase EGFR tyrosine phosphorylation by activating an as yet uncharacterized tyrosine kinase or by inhibiting a protein tyrosine phosphatase. Whether or not calcium activates the EGFR kinase activity per se, it does activate EGFR signal transduction by inducing association of the receptor with the downstream signaling proteins Shc, Grb2, and Sos1.

Although calcium activation of growth factor receptor signaling is likely to involve other receptor tyrosine kinases, we have also found evidence for its specificity. For example, KCl treatment of PC12 cells does not induce tyrosine phosphorylation of the insulin receptor (unpublished data) or the NGF receptor Trk (Fig. 5B). In addition, the observed effects of calcium appear to be specific to certain cell types. We have not detected inducible tyrosine phosphorylation of the EGFR or Shc in response to KCl treatment of cortical neurons, where calcium influx may activate Ras through a specific CaM-binding GEF that is not detectable in PC12 cells (69). In other studies, ionomyucin treatment of A431 cells and extracellular calcium addition to keratinocytes inhibited EGFR tyrosine phosphorylation (53, 54). These inhibitory effects may involve calcium activation of serine/threonine kinases, since phosphorylation of the EGFR on serine and threonine residues can down-regulate both EGF binding and receptor tyrosine kinase activity (55). The cell specificity of calcium effects on EGFR tyrosine phosphorylation suggests that the stimulation we observe is not simply due to a conformational change in the EGFR induced by calcium ions, as has been described in vitro

FIG. 5. Calcium induction of ErbB2 but not Trk tyrosine phosphorylation. PC12 cells were treated with 50 mM KCl (lanes K), 100 ng of NGF per ml (lanes N), or 10 ng of EGF per ml (lanes E) for 3 min (lanes C, controls). Lysates were immunoprecipitated with anti-EGFR pAb or anti-ErbB2 (A) or anti-Trk (B) antibody, and precipitated proteins (IP) were analyzed by immunoblotting with anti-Tyr(p) antibodies. Positions of migration of prestained molecular size markers (kDa) are shown.

FIG. 6. Calcium induction of EGFR signal transduction. Membrane depolarization leads to calcium influx through VSCCs. This leads to tyrosine phosphorylation of the EGFR, possibly by activating a cytoplasmic tyrosine kinase such as Src. Trans-phosphorylation of the EGFR by the cytoplasmic tyrosine kinase may activate the receptor to autophosphorylate on the same sites that would be phosphorylated in response to EGF binding. Calcium-induced tyrosine phosphorylation of the EGFR leads to its association with the downstream adaptor proteins Shc and Grb2, which allows localization of the guanine nucleotide exchange factor Sos1 to the plasma membrane, where it can activate Ras and initiate signaling to MAPK.

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in the presence of millimolar concentrations of Mg^{2+} or Mn^{2+} (56). The distinct effects of increased cytosolic calcium on EGFR tyrosine phosphorylation in different cell types may reflect the differential expression of calcium-responsive signaling intermediates or distinct modes of calcium entry into the cells, which can generate different signaling responses (5).

Stimulation of growth factor receptor signaling pathways in response to VSCC activation has general implications for how calcium signals may be transduced into biochemical responses in neurons. EGF and its receptor are expressed in a number of areas in the nervous system (48, 57), and expression of the EGFR family members ErbB2 (c-neu), ErbB3, and ErbB4 has also been detected in brain (58–60). In addition, a family of ligands that bind the ErbB proteins, the neuregulins, has recently been discovered, which can act as trophic factors in the nervous system (61). This widespread expression of EGFR family signaling machinery in the nervous system suggests that it could be used in response to activity-dependent calcium influx via the mechanism described here. For example, one long-term response to membrane depolarization-induced calcium influx is enhanced survival of certain neuronal populations, such as cerebellar granule cells (62, 63). EGF itself has been shown to be a survival factor for certain types of neurons as well, including cerebellar granule neurons (47, 64, 65). Thus, one mechanism by which neuronal activity may enhance neuronal survival may be through calcium activation of EGFR signaling pathways. Calcium stimulation of growth factor signaling pathways may be a general mechanism for activity-dependent regulation of survival and trophic responses in the nervous system (62, 66).

Note Added in Proof. While this manuscript was in press, others also observed that calcium influx leads to Shc phosphorylation in PC12 cells. The Shc phosphorylation event was shown to be mediated by Src (70) and/or a novel 112-kDa calcium-responsive tyrosine kinase, PYK2 (71).

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Related Adhesion Focal Tyrosine Kinase and the Epidermal Growth Factor Receptor Mediate the Stimulation of Mitogen-activated Protein Kinase by the G-protein-coupled P_{2Y2} Receptor

PHORBOL ESTER OR $[Ca^{2+}]_i$ ELEVATION CAN SUBSTITUTE FOR RECEPTOR ACTIVATION*

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The activation of growth factor receptors and receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G-proteins) can increase mitogen-activated protein (MAP) kinase activity in many cells. Previously, we demonstrated that the activation of G-protein-coupled P_{2Y2} receptors by extracellular ATP and UTP stimulated MAP (p42 ERK2) kinase by a mechanism that was dependent on the elevation of $[Ca^{2+}]_i$ and the activation of related adhesion focal tyrosine kinase (RAFTK) (also called PYK2, CAKβ, and CADTK) and protein kinase C (PKC). Here, we examine further the signaling cascade between the P_{2Y2} receptor and MAP kinase. MAP kinase was transiently activated by exposure of PC12 cells to UTP. UTP, ionomycin, and phorbol ester (phorbol 12-myristate 13-acetate) increased MAP kinase activity and also promoted the tyrosine phosphorylation of RAFTK, the epidermal growth factor (EGF) receptor, SHC, and p120^{cbl}. Downregulation of PKC and inhibition of the elevation of $[Ca^{2+}]_i$, conditions that block the activation of MAP kinase, also blocked the increases in the tyrosine phosphorylation of RAFTK and the EGF receptor. AG1478, a tyrphostin selective for the EGF receptor, reduced the activation of MAP kinase, the tyrosine phosphorylation of SHC, the association of Grb2 with SHC, and the tyrosine phosphorylation of the EGF receptor and p120^{cbl} but did not block the tyrosine phosphorylation of RAFTK. The similar effects of UTP, ionomycin, and phorbol 12-myristate 13-acetate (PMA) on these signaling proteins demonstrate that the two signaling molecules from phosphatidylinositol 4,5-bisphosphate hydrolysis ($[Ca^{2+}]_i$, from inositol 1,4,5-trisphosphate production, and diacylglycerol) can individually initiate the activation of MAP kinase in an EGF receptor-dependent manner. These results demonstrate that the P_{2Y2} receptor-mediated transactivation of the EGF receptor occurs at a point downstream of RAFTK and indicate that the EGF receptor is required for P_{2Y2} receptor-mediated MAP kinase activation. Although P_{2Y2} and EGF receptors may both activate a similar multiprotein signaling cascade immediately upstream of MAP kinase, the P_{2Y2} receptor appears to uniquely utilize $[Ca^{2+}]_i$, PKC, and, subsequently, RAFTK.

Recent studies have demonstrated that growth factor receptors and G-protein-coupled receptors (GPCRs)[1] may both activate the same signal transduction molecules and utilize the same signaling cascades in cells. One of the most common signaling events mediated by both types of receptors is the activation of mitogen-activated protein (MAP) kinase, although portions of the signaling cascade between the receptors and MAP kinase can be different for growth factor receptors and GPCRs, particularly pertaining to the involvement of members of the PKC family of proteins. Previously, we examined the effects of extracellular nucleotides on PC12 cells (1). Extracellular nucleotides can bind to P_2-type purinoceptors, which constitute a large family of receptors that are either ion channels (P_{2X} subtypes) or else coupled to G-proteins (P_{2Y} subtypes) and which vary in their tissue distribution (2, 3). Previously, we observed that both extracellular ATP and UTP increased MAP kinase activity in a nucleotide concentration-dependent manner in PC12 cells, which also respond to EGF and NGF with increases in MAP kinase activity. For both ATP and UTP, the EC_{50} value was ~25 μM, and 100 μM promoted nearly a maximal effect (1). These results were consistent with the nucleotide-dependent activation of the P_{2Y2} receptor. This purinoceptor was previously designated P_{2U}, because it does not discriminate between ATP and UTP on the basis of potency. The P_{2Y2} receptor-mediated activation of MAP kinase involved the elevation of $[Ca^{2+}]_i$, the activation of PKC, and the tyrosine phosphorylation and activation of RAFTK (1). In contrast, these signaling events did not appear to play a major role in the mechanism of EGF-initiated increase in MAP kinase activity.

The effects of a number of GPCRs have been reported to involve increases in tyrosine phosphorylation. Signaling proteins such as SHC (4, 5) and members of the Src kinase family may contribute to GPCR-mediated activation of signaling pathways, including MAP kinase (4–11). In addition, the Gβγ subunit of heterotrimeric G-proteins can mediate MAP kinase activation (5, 12, 13). GPCRs also can transactivate growth factor receptors, including the EGF receptor and the PDGF receptor (4, 9–11). Several reports suggest that the EGF receptor and other proteins may serve as a scaffolding structure or as an adaptor protein to which other signaling proteins may be recruited in response to GPCR signaling (5, 14). Proteins serving in this capacity can localize proteins to a particular region

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[1] The abbreviations used are: GPCR, G-protein-coupled receptor; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; DAG, diacylglycerol; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; NGF, nerve growth factor; RAFTK, related adhesion focal tyrosine kinase; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, acetoxymethyl ester; MAP, mitogen-activated protein; PDGF, platelet-derived growth factor.

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of the cell or a microdomain of a membrane. This protein localization may allow for their biochemical function, which may include substrate phosphorylation or product generation, to be performed at a physiologically relevant site.

In the present report, we continued our examination of the activation of MAP kinase by UTP and other stimuli and focused particular attention on the involvement of RAFTK and the potential involvement of the EGF receptor. RAFTK (also called PYK2, CAKβ, and CADTK) is a protein-tyrosine kinase that is activated by elevations of $[Ca^{2+}]_i$ or by phorbol esters, and it is involved in the activation of MAP kinase by some stimuli (7, 15). Consistent with our previous results, our new studies suggest a role for RAFTK in the activation of MAP kinase by UTP. In addition, the EGF receptor appears to be directly involved in the signaling cascade that leads to MAP kinase activation by UTP, ionomycin, and PMA. A tyrphostin (AG1478) that blocks the EGF receptor tyrosine kinase also blocks UTP-, PMA-, and ionomycin-promoted MAP kinase activity, SHC tyrosine phosphorylation, and Grb2 association with SHC. AG1478 also blocks the UTP- and ionomycin-initiated tyrosine phosphorylation of other signaling proteins downstream of the EGF receptor, such as p120^{cbl}, but does not block RAFTK tyrosine phosphorylation. The tyrosine phosphorylation of RAFTK is increased by UTP and ionomycin more than by EGF. The results suggest that RAFTK activation is upstream of the EGF receptor and that both proteins are central to the activation of MAP kinase by the G-protein-coupled P_{2Y2} receptor. Although there are similarities in the signaling cascades involved in MAP kinase activation initiated by EGF and UTP, notably EGF receptor and SHC tyrosine phosphorylation, RAFTK and PKC are uniquely involved in the P_{2Y2} receptor-mediated cascade.

MATERIALS AND METHODS

Reagents—All chemicals were reagent grade or better. Dulbecco's modified Eagle's medium and PMA were obtained from Life Technologies, Inc. Calf serum, horse serum, UTP, and ATP were purchased from Sigma, EGF (catalog no. 01-107) from Upstate Biotechnology, Inc. (Lake Placid, NY), and NGF (2.5S) from Boehringer Mannheim. [^{32}P]ATP (specific activity, 3000 Ci/mmol) was purchased from NEN Life Science Products. Anti-phosphotyrosine antibody was a generous gift of Dr. Tom Roberts (Dana Farber Cancer Center, Boston, MA). Polyclonal anti-RAFTK antibody was produced as described previously (16) and was a generous gift of Dr. Hava Avraham (Beth Israel Deaconess Medical Center, Boston, MA). Polyclonal anti-SHC (S14630) and monoclonal anti-SHC (S14620) antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-EGF receptor antibody (SC-03), anti-Cbl (SC-170), anti-Grb2 (SC-255), and anti-ERK2 (SC-154) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). AG1478 (product 658552) was purchased from Calbiochem. BAPTA-AM was purchased from Molecular Probes, Inc. (Eugene, OR).

Cell Culture—PC12 cells were grown in 100-mm-diameter dishes at 37 °C in a 95% air, 5% CO_2 mixture in Dulbecco's modified Eagle's medium with 5% calf serum and 5% horse serum. Cells were used at or near confluence. Cells were maintained overnight in low serum medium (Dulbecco's modified Eagle's medium plus 0.05% calf serum, 0.05% horse serum) in all experiments. Cells were exposed to stimuli by exchanging this medium with medium containing stimuli or vehicle (Me$_2$SO or water). Cells in which PKC was down-regulated were exposed to 1 μM PMA or vehicle (0.06% Me$_2$SO) overnight (~16 h). In some experiments, cells were pretreated with AG1478 or vehicle (Me$_2$SO) for 15–30 min, followed by various treatment conditions as indicated. In experiments designed to block the elevation in $[Ca^{2+}]_i$, serum-starved cells were exposed to 10 μM BAPTA-AM or vehicle (0.1% Me$_2$SO) for 30 min and then switched to low serum medium with or without 5 mM EGTA with or without stimuli or vehicle for 5 min.

Immunoprecipitation and Western Blotting—After cells were exposed to a stimulatory agent for the designated time, the cells were washed twice with an ice-cold buffered saline solution (137 mM NaCl, 20 mM Tris base, 1 mM EGTA, 1 mM EDTA, 0.2 mM vanadate, pH 7.5), and were lysed in lysis buffer (137 mM NaCl, 20 mM Tris base (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40) contain-

ing the following phosphatase and protease inhibitors: 1 mM vanadate, 1 mM ZnCl$_2$, 4.5 mM sodium pyrophosphate, 2 mg/ml NaF, 2 mg/ml β-glycerophosphate, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, and 2 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride. The lysates were vortexed thoroughly and centrifuged at 16,000 × *g* (Eppendorf 5414 microcentrifuge). The cleared supernatants were transferred to fresh microcentrifuge tubes. In some experiments, a portion (5–10% of the volume) of the lysate was removed and combined with an equal volume of 2× sample buffer (62.5 mM Tris, pH 6.8, 10% (v/v) glycerol, 6.25% (v/v) SDS, 0.72 N β-mercaptoethanol; bromphenol blue for color). The remainder was incubated at 4 °C for 3 h or overnight with anti-ERK2 (1 μg/ml), polyclonal anti-SHC (1 μg/ml), anti-EGF receptor (1.5 μg/ml), anti-RAFTK (5 μl), or anti-Tyr(P) antibody (~6 μg/ml), plus protein G-Sepharose (4 mg/ml) with the anti-RAFTK antibody and protein A-Sepharose (4 mg/ml) with the other antibodies. At the end of the incubation, the immunoprecipitates were collected by centrifugation. The immunoprecipitates were washed two times in ice-cold phosphate-buffered saline (137 mM NaCl, 15.7 mM NaH$_2$PO$_4$, 1.47 mM KH$_2$PO$_4$, 2.68 mM KCl, 1% Nonidet P-40, pH 7.4); one time in 0.1 M Tris (pH 7.5), 0.5 M LiCl; and two times in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5). All wash solutions contained 0.2 mM vanadate. The majority of the TNE was removed, the remaining volume was diluted with 70 μl of 2× sample buffer, and the samples were boiled for 5–10 min. The immunoprecipitated proteins and the lysate fractions were subjected to electrophoresis or stored at −80 °C prior to electrophoresis.

Samples were subjected to electrophoresis on a SDS-polyacrylamide separating gel with a 3% stacking gel. The separating gel was 12% for anti-SHC immunoprecipitates, and 7% for all others. Proteins were transferred to 0.2 μm pore-size nitrocellulose filters, and the filters were blocked with TBS (20 mM Tris (pH 7.6), 137 mM NaCl, 2% (w/v) BSA for 1 h. The filters were washed in TTBS (TBS, 0.2% (v/v) Tween 20) three times. The nitrocellulose filters were exposed to blotting antibodies in TTBS, 1% bovine serum albumin for ~16 h at 4 °C. The filters were washed three times in TTBS and exposed to goat anti-rabbit or goat anti-mouse horseradish peroxidase (Boehringer Mannheim) at a 1:10,000 dilution in TTBS plus 1% bovine serum albumin for 1 h. All washes and exposure to the secondary antibody were performed at room temperature. Filters were washed three times with TTBS and twice with TBS and were visualized on x-ray film (Eastman Kodak Co.) using a chemiluminescence system (Amersham Pharmacia Biotech or NEN Life Science Products). In some experiments, the filters were stripped of antibodies by exposing them to 62.5 mM Tris (pH 6.8), 0.1 M β-mercaptoethanol, 2% (w/v) SDS at 70 °C for 40 min. The stripped filters were washed several times in TTBS, washed once in TBS, blocked with TBS plus 2% bovine serum albumin for 1 h, and reprobed with antibody overnight. Blots were then treated as described above.

ERK2/MAP Kinase Activity—ERK2 was immunoprecipitated from PC12 cells by incubating cleared lysates with anti-ERK2 antibody and protein A-Sepharose beads for 3–4 h (see above). The immunoprecipitates were washed two times with RIPA lysis buffer containing 1 mM vanadate and two times with kinase buffer (50 mM Tris, pH 7.5, 5 mM MgCl$_2$). Immunoprecipitates were resuspended in a final volume of 50 μl of kinase buffer containing myelin basic protein (200 μg/ml kinase buffer), and the kinase assay was initiated with the addition of 40 μM ATP plus [^{32}P]ATP (1 μCi). After 30 min, the supernatant was added to an equal volume of 2× sample buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis using a 15% separating gel. The phosphorylated myelin basic protein was quantified using a molecular imager system (Bio-Rad GS-363). For each independent experiment, each condition was assayed in duplicate or triplicate.

Data—Immunoblots similar to those shown in the figures were obtained in two or more independent experiments. The numbers (*n*) of independent assays of ERK2 activity were as noted below.

RESULTS

Time Dependence of UTP on MAP Kinase Activity—An established paradigm in PC12 cells is that agents that cause proliferation (*e.g.* EGF) produce short lived increases in MAP kinase, while agents that cause differentiation (*e.g.* NGF) produce much longer lived increases that are sustained for hours (17, 18). Previously, we observed that UTP did not produce a measurable increase in MAP kinase activity at 1 min but produced a substantial increase after a 5-min exposure (1). Therefore, we examined the degree to which the UTP-promoted increase in MAP kinase activity was sustained as a function of
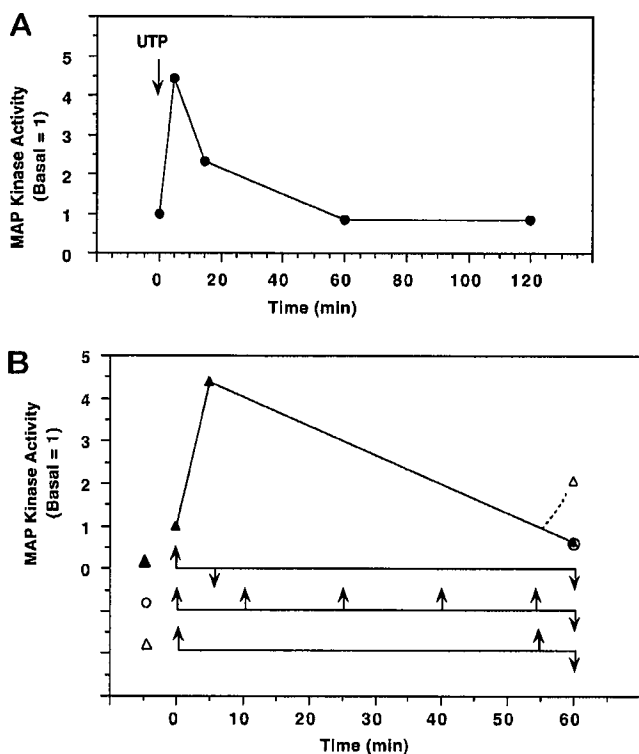
A

B

FIG. 1. **Time course of the UTP-promoted increase in MAP kinase activity.** MAP kinase activity of anti-ERK2 immunoprecipitates was measured using a substrate (myelin basic protein) phosphorylation assay. *A*, cells were exposed UTP (100 μM) for 5–120 min. For each sample, the cells were exposed to a single UTP-containing solution from time 0 to the time at which the sample was collected. The increase in MAP kinase activity was greatest after a 5-min exposure, and the peak activity commenced its return to basal levels within 15 min. *B*, cells were exposed to UTP (100 μM) at time 0, and some samples were collected after 5 min. Other samples were collected at 60 min after various additional exposures to UTP-containing solutions. The switches to UTP-containing solutions and the collection of samples are represented by *upward* and *downward arrows*, respectively. After reaching a peak at 5 min, the MAP activity returned to basal levels after 60 min regardless of whether cells were exposed to UTP by a single exposure to UTP-containing media or by multiple exposures to UTP-containing media. However, if cells were exposed to fresh UTP-containing media for a 5-min period between 55 and 60 min after a single exposure at time 0, there was an increase in MAP kinase activity. These results are consistent with the desensitization of the P$_{2Y2}$ receptor in the presence of UTP and the resensitization of the receptor upon consumption of the added UTP.

time in an extended time course. UTP promoted a transient increase in MAP kinase activity; the peak increase was at 5 min, and the activity returned to basal levels between 15 and 60 min (Fig. 1*A*). These experiments were performed by exposing cells to a single presentation of a UTP (100 μM)-containing solution for various periods of time. Since many cells have ectonucleotidases and ecto-ATPases that can hydrolyze extracellular nucleotides, it was possible that the lack of a sustained MAP kinase activation was due to the consumption of ligand, rather than to an intrinsic deactivation of MAP kinase. Therefore, in order to maintain the UTP concentration and study the time-dependent alterations in MAP kinase activity, we modified the protocol to include multiple solution switches during a 60-min time course. In these experiments, the cells were exposed to a fresh UTP (100 μM)-containing solution four times during a 55-min period and then once again for 5 min prior to the collection of the 60-min time point (Fig. 1*B*). However, even under these conditions, the UTP-promoted MAP kinase activity was not sustained, and it returned to basal levels within 60 min. The MAP kinase activity returned to a similar level after
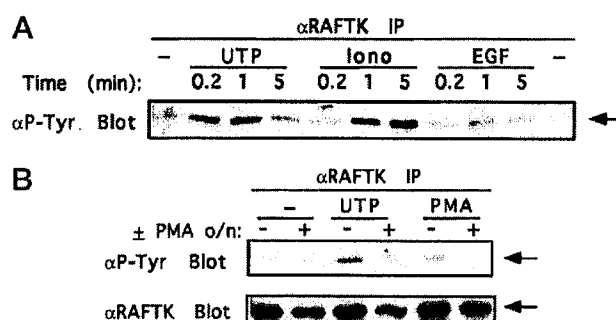
A

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FIG. 2. **Time and PKC dependence of RAFTK tyrosine phosphorylation in cells exposed to UTP and other stimuli.** *A*, cells were exposed to UTP (100 μM), ionomycin (10⁻⁶ M), or EGF (100 ng/ml) for 0.2–5 min. RAFTK was immunoprecipitated (*IP*) using anti-RAFTK antibody and immunoblotted using anti-Tyr(P) (*α-P-Tyr*) antibody. UTP produced a rapid increase in RAFTK tyrosine phosphorylation, which was greatest between 0.2 and 1 min. The peak increase in cells exposed to ionomycin was relatively delayed and occurred between 1 and 5 min. EGF promoted a relatively small increase in the tyrosine phosphorylation of RAFTK. *B*, cells were treated with PMA (1 μM) or vehicle (Me$_2$SO) overnight, and then exposed to vehicle (water), UTP (100 μM), or PMA (200 nM) for 1 min. Proteins were immunoprecipitated using anti-RAFTK antibody and sequentially immunoblotted using anti-Tyr(P) (*top*) and anti-RAFTK (*bottom*) antibodies. The responses to UTP and PMA were reduced in cells in which PKC was down-regulated by long term exposure to PMA.

exposure to a single UTP-containing solution for 60 min. However, if cells were exposed continuously to a single UTP-containing solution for 55 min followed by exposure to a fresh solution of UTP for an additional 5 min, there was an increase in MAP kinase activity (Fig. 1*B*). These results suggest that UTP is degraded when it is exposed to cells for an extended period of time and that the P$_{2Y2}$ receptor can resensitize to UTP under these conditions. In addition, the activation of MAP kinase is not maintained even under conditions designed to maintain the UTP concentration.

Time Course of RAFTK Tyrosine Phosphorylation—The tyrosine phosphorylation and activation of RAFTK (PYK2) is upstream of MAP kinase (7, 8, 15). Therefore, the time course of RAFTK tyrosine phosphorylation was examined in PC12 cells exposed to UTP and other stimuli (Fig. 2*A*). The tyrosine phosphorylation of RAFTK increased after 15-s and 1-min exposure to UTP. The phosphorylation after a 5-min exposure usually was reduced compared with the 1-min exposure. Ionomycin produced large increases in RAFTK tyrosine phosphorylation after a 1- and 5-min exposure. EGF produced increases in RAFTK tyrosine phosphorylation, but these increases were much smaller than those produced by UTP and ionomycin. These results are consistent with an elevation of [Ca^{2+}]$_i$ upstream of RAFTK activation, in agreement with our previous results in which the tyrosine phosphorylation of RAFTK and the activation of MAP kinase by UTP and ionomycin were reduced in cells in which [Ca^{2+}]$_i$ elevation was reduced by loading the cells with BAPTA-AM (1). The results show that the UTP-dependent tyrosine phosphorylation of RAFTK occurs at times (≤1 min) that precede the peak increase in MAP kinase activity.

PKC Down-regulation Reduces the UTP-dependent Tyrosine Phosphorylation of RAFTK—To determine whether PKC was involved in the UTP-initiated tyrosine phosphorylation of RAFTK, cells were treated overnight with PMA (1 μM) to down-regulate protein kinases C. This treatment substantially reduced the tyrosine phosphorylation of RAFTK by UTP (100 μM, 1 min) and by acute exposure to PMA (200 nM, 1 min) (Fig. 2*B*). This is in agreement with the fact that RAFTK can be stimulated by PKC activation/DAG production as well as by an elevation of [Ca^{2+}]$_i$ (15). In addition, since the activation of
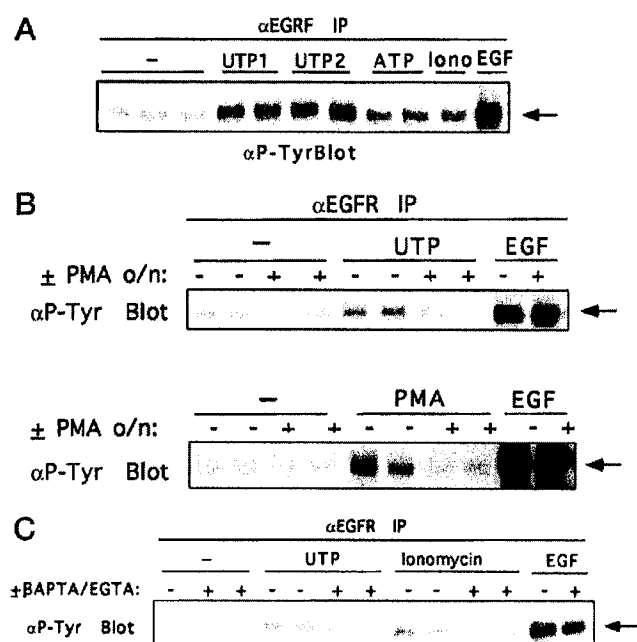
A

B

C

Fig. 3. **UTP and other stimuli increase the tyrosine phosphorylation of the EGF receptor in a $[Ca^{2+}]_i$- and PKC-dependent manner.** Cells were exposed to UTP (100 μM), ATP (100 μM), ionomycin (10^{-6} M), PMA (200 nM), and EGF (100 ng/ml), as indicated. After various periods of exposure, the cells were lysed, and the EGF receptor was immunoprecipitated (IP) using an anti-EGF receptor antibody. The immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted using anti-Tyr(P) (α-P-Tyr) antibody. *A*, UTP (1 or 2 min, as indicated), ATP (1 min), and ionomycin (1 min) promoted significant increases in the tyrosine phosphorylation of the EGF receptor. EGF (1 min) produced much a larger increase in tyrosine phosphorylation than any of the other stimuli. Each lane is an immunoprecipitate from a separate dish of cells treated as indicated. *B*, cells were treated with 1 μM PMA (+) or Me_2SO (−) overnight, and then exposed to vehicle (−), UTP (5 min), PMA (5 min), or EGF (1 min). Each set of *lanes* (*upper* and *lower*) are the results from a separate experiment conducted on different days. The responses to UTP and PMA were reduced in cells in which PKC was down-regulated by long term exposure to PMA. *C*, cells were pretreated with either Me_2SO (−) or BAPTA-AM (+) for 30 min and then exposed to vehicle (5 min), UTP (5 min), ionomycin (5 min), or EGF (1 min). Cells exposed to BAPTA-AM were also exposed to EGTA during their exposure to stimuli. The effects of UTP and ionomycin on the tyrosine phosphorylation of the EGF receptor were reduced in BAPTA-AM/EGTA-treated cells.

MAP kinase by UTP is dependent on PKC (1), it is consistent with the involvement of RAFTK in the UTP-promoted activation of MAP kinase.

UTP and Other Stimuli Increase the Tyrosine Phosphorylation of the EGF Receptor—Previously, we observed that UTP, ATP, and other stimuli produced an increase in the tyrosine phosphorylation of a ~160-kDa protein (1) the size of the EGF receptor. Since ligands of some G-protein-coupled receptors, $G\beta\gamma$, and increases in $[Ca^{2+}]_i$ (5, 19, 20) can activate the EGF receptor, we examined whether UTP and other stimuli promoted the tyrosine phosphorylation of the EGF receptor, which was immunoprecipated using an anti-EGF receptor antibody. UTP, ATP, and ionomycin produced significant increases in the tyrosine phosphorylation of the EGF receptor within 1 min of exposure to the cells (Fig. 3A). PMA also increased the level of EGF receptor tyrosine phosphorylation (Fig. 3B), although generally it was somewhat less effective than the increases produced by UTP or ionomycin. Not surprisingly, the effects of all of these stimuli on EGF receptor tyrosine phosphorylation were much less than those produced by maximum concentrations (100 ng/ml) of EGF. These studies suggest that ionomycin,

which increases $[Ca^{2+}]_i$; PMA, which mimics DAG; and UTP, a ligand that increases both $[Ca^{2+}]_i$ and DAG production, all promote an increase in the tyrosine phosphorylation of the EGF receptor. These results are consistent with the transactivation of the EGF receptor by these stimuli (see below).

Since the activation of MAP (ERK2) kinase by UTP and PMA was diminished in cells in which PKC was down-regulated by treatment of the cells with PMA overnight (1), the effect of this treatment on the tyrosine phosphorylation of the EGF receptor was examined. Down-regulation of PKC reduced the ability of both UTP and PMA to promote increases in the tyrosine phosphorylation of the EGF receptor (Fig. 3B). The activation of MAP kinase by UTP and ionomycin also was decreased in cells exposed to conditions that block the elevation of $[Ca^{2+}]_i$ (1). In a similar manner, the UTP- and ionomycin-promoted increases in the EGF receptor tyrosine phosphorylation were blocked in BAPTA-AM-loaded EGTA-treated cells (Fig. 3C). Thus, increases in the tyrosine phosphorylation of the EGF receptor are blocked by conditions that diminish the activation of MAP kinase by UTP, increases in $[Ca^{2+}]_i$, and the activation of PKC. Increases in the tyrosine phosphorylation of RAFTK also are reduced by down-regulation of PKC (Fig. 2B) and treatment of cells with BAPTA-AM and EGTA (1). These results indicate that transactivation of the EGF receptor and the activation of MAP kinase are both downstream of the activation of the G-protein-coupled P_{2Y2} receptor, but they do not demonstrate whether these downstream events occur in parallel or in a linear and dependent manner.

Inhibition of the EGF Receptor Blocks MAP Kinase Activation by UTP and Other Stimuli—To determine whether the EGF receptor was involved in the stimulation of MAP kinase by extracellular nucleotides and other stimuli, the EGF receptor tyrosine kinase activity was blocked by treating cells with AG1478, a tyrphostin that is selective for the EGF receptor (21). The concentration dependence of AG1478 was examined using cell lysates immunoblotted with anti-Tyr(P) antibody. The EGF-dependent tyrosine phosphorylation the EGF receptor was reduced by AG1478 in a concentration-dependent manner between 10 and 300 nM (Fig. 4A). Ionomycin and UTP increased the tyrosine phosphorylation of a band that co-migrated with the EGF receptor. This was observable only faintly for ionomycin- and UTP-treated cells at this length of exposure of the immunoblot to enhanced chemiluminescence solutions but was more detectable at longer times of exposure (not shown). The phosphorylation of this band was decreased in cells exposed to AG1478.

EGF, UTP, and ionomycin also produced an increase in the tyrosine phosphorylation of a ~42-kDa band that co-migrated with ERK2, and this phosphorylation was nearly completely reduced by 100 nM AG1478 (Fig. 4A). AG1478 did not block the NGF-stimulated tyrosine phosphorylation of this 42-kDa protein (not shown), consistent with the lack of effect of AG1478 on p140^{trk}, the receptor for NGF. These results suggest that increases in MAP kinase by UTP, ionomycin, and PMA are mediated by the EGF receptor. To examine this in a more quantitative and definitive manner, the effects of AG1478 on ERK2 activity also were measured using an *in vitro* kinase (substrate phosphorylation) assay of anti-ERK2 immunoprecipitates. AG1478 did not significantly reduce the basal (unstimulated) ERK2 activity (Fig. 4B). However, the stimulations of ERK2 by UTP, ionomycin, and PMA, were blocked to the same degree (60–80%) as was that of EGF (Fig. 4B), suggesting that the effects of these three agents were mediated by the activation of the EGF receptor at a point upstream of MAP kinase. This is consistent with the stimulation of EGF receptor tyrosine phosphorylation by UTP, ionomycin, and PMA (Fig. 3).
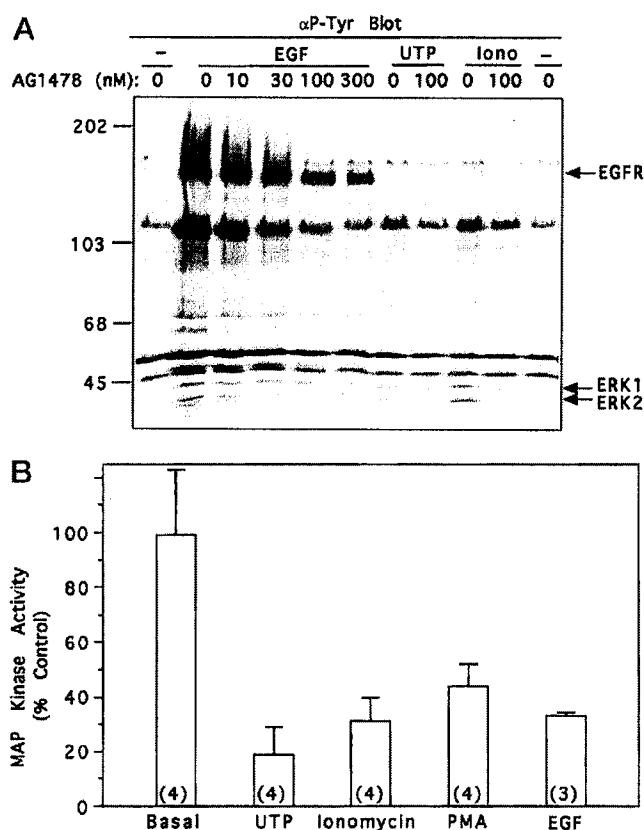
A

B

FIG. 4. **AG1478 blocks the tyrosine phosphorylation of the EGF receptor and the activation of MAP kinase by UTP, ionomycin, PMA, and EGF.** *A*, cells were pretreated with vehicle (*0*) or different concentrations of AG1478 (10–300 nM) for 15 min and then exposed to EGF (100 ng/ml, 1 min), UTP (100 μM, 5 min), and ionomycin (10^{-6} M, 5 min). Cell lysates were immunoblotted using anti-Tyr(P) (α-*P-Tyr*) antibody. UTP and ionomycin increase the tyrosine phosphorylation of multiple proteins, including those that co-migrate with the EGF receptor (*upper arrow*), ERK1 (*middle arrow*), and ERK2 (*lower arrow*). The UTP- and ionomycin-promoted tyrosine phosphorylation of proteins that co-migrate with the EGF receptor was greater in Western blots that were exposed to enhanced chemiluminescence solution for a longer period of time (not shown). AG1478 blocked the EGF-promoted tyrosine phosphorylation of the EGF receptor and other proteins in a concentration-dependent manner. AG1478 (100 nM) also reduced the UTP- and iono-mycin-promoted tyrosine phosphorylation of proteins that co-migrated with the EGF receptor, ERK1, and ERK2. *B*, cells were pretreated with AG1478 (300 nM) or vehicle for 30 min and then exposed to vehicle (*Basal*), UTP (100 μM), ionomycin (10^{-6} M), PMA (200 nM), and EGF (100 ng/ml) for 5 min. MAP kinase *in vitro* phosphorylation assays were performed using anti-ERK2 immunoprecipitates. For each stimulus, the activity obtained in the presence of AG1478 was compared with that obtained in the absence of AG1478. AG1478 did not reduce the basal (unstimulated) MAP kinase activity, but the effects of EGF, UTP, ionomycin, and PMA were inhibited by 60–80%.

AG1478 Blocks the Tyrosine Phosphorylation of p120^{cbl} by UTP and Other Stimuli—p120^{cbl} is a 120-kDa protein that is phosphorylated on tyrosine in EGF-treated PC12 cells. Exposure of PC12 cells to UTP promoted the tyrosine phosphorylation of p120^{cbl} in a time-dependent manner. Phosphorylation was observable after 1 and 5 min of UTP treatment (Fig. 5*A*). The phosphorylation was not readily detectable after a 15-s exposure to UTP, although there is a large increase in the tyrosine phosphorylation of p120^{cbl} in PC12 cells treated for 15 s with EGF (22). Ionomycin also promoted an increase in the tyrosine phosphorylation of p120^{cbl} (Fig. 5*B*). The degree of p120^{cbl} phosphorylation initiated by UTP and ionomycin were significantly less than that produced by EGF, and the effects of all three stimuli were reduced by AG1478 (Fig. 5*B*).

A

B

C
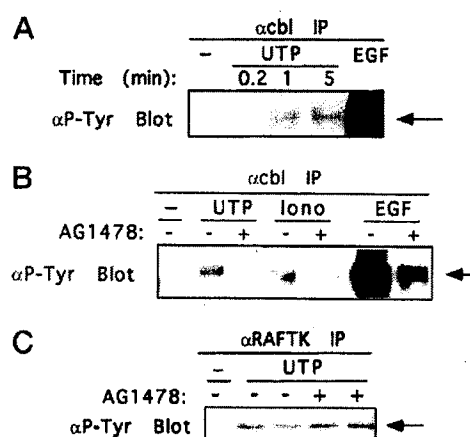
FIG. 5. **The UTP-promoted tyrosine phosphorylation of p120^{cbl} and RAFTK are downstream and upstream, respectively, of the EGF receptor.** *A*, cells were exposed to UTP (100 μM) for 15 s to 5 min or EGF (100 ng/ml) for 1 min. p120^{cbl} was immunoprecipitated using anti-Cbl antibody, and immunoblotted using anti-Tyr(P) (α-*P-Tyr*) antibody. There was an increase of the tyrosine phosphorylation of p120^{cbl} in cells exposed to UTP for 1 and 5 min. The EGF-dependent increase in the tyrosine phosphorylation of p120^{cbl} was larger than that produced by UTP and is overexposed in this immunoblot in order to highlight the effects of UTP. *B*, cells were pretreated with 200 nM AG1478 (+) or vehicle (−) for 20 min and then exposed to UTP (100 μM), ionomycin (10^{-6} M), and EGF (100 ng/ml) for 5 min. p120^{cbl} was immunoprecipitated using anti-Cbl antibody, and immunoblotted using anti-Tyr(P) antibody. UTP and ionomycin increased the tyrosine phosphorylation of p120^{cbl}. This was reduced in cells exposed to AG1478, suggesting that the effects of UTP and ionomycin on p120^{cbl} were downstream of the activation of the EGF receptor. *C*, cells were pretreated with 100 nM AG1478 (+) or vehicle (−) for 30 min and then exposed to UTP (100 μM) for 1 min. RAFTK was immunoprecipitated using anti-RAFTK antibody and immunoblotted using anti-Tyr(P) antibody. AG1478 did not block the UTP-promoted tyrosine phosphorylation of RAFTK.

AG1478 Does Not Block the UTP-stimulated Tyrosine Phosphorylation of RAFTK—To determine whether the UTP-dependent activation of RAFTK was upstream or downstream of the EGF receptor, the effect of AG1478 on RAFTK tyrosine phosphorylation was examined. The UTP-initiated phosphorylation of RAFTK was not affected by pretreatment of the cells with either 100 nM AG1478 (Fig. 5*C*) or 300 nM AG1478 (not shown). These results suggest that RAFTK is upstream of the activation of the EGF receptor. Along with the inhibitory effects of AG1478 on MAP kinase (Fig. 4), these results suggest that the activation of the EGF receptor is between RAFTK and MAP kinase in the signaling cascade promoted by UTP, iono-mycin, and PMA.

AG1478 Blocks the Tyrosine Phosphorylation of SHC by UTP, Ionomycin, PMA, and EGF—Previously, we demonstrated that UTP and other stimuli produced an increase in the tyrosine phosphorylation of SHC, and this was observable after exposure of cells to UTP for 1–5 min. Grb2 was co-immunoprecipitated in the anti-SHC immunoprecipitate of PC12 cells exposed to UTP and other stimuli, and the relative amount of immunoprecipitable Grb2 was proportional to the level of tyrosine phosphorylation of SHC produced by UTP, ionomycin, PMA, NGF, and EGF (1). This was consistent with Grb2 binding via its Src homology 2 domain to tyrosine residues on SHC. To determine if the tyrosine phosphorylation of SHC was dependent on the intrinsic tyrosine kinase activity of the EGF receptor, the effects of various stimuli on SHC phosphorylation were examined in the presence and absence of AG1478.

The tyrosine phosphorylation of SHC by UTP, ionomycin, PMA, and EGF, but not by NGF, was blocked by pretreatment of cells with AG1478 (Fig. 6, *middle panel*). The association of

FIG. 6. **UTP and other stimuli promote the tyrosine phosphorylation of SHC and its association with Grb2 and the EGF receptor, and this is blocked by AG1478.** Cells were pretreat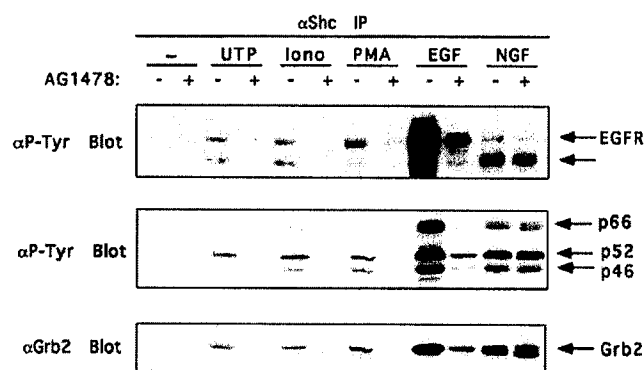ed with AG1478 (300 nM, 30 min) and then exposed to UTP (100 μM), ionomycin (10^{-6} M), PMA (200 nM), EGF (100 ng/ml), and NGF (100 ng/ml) for 5 min. Proteins were immunoprecipitated using anti-SHC antibody. SHC (*middle panel*) and proteins of larger mass (*upper panel*) were immunoblotted using anti-Tyr(P) (α-P-Tyr) antibody, and Grb2 was immunoblotted using an anti-Grb2 antibody (*lower panel*). The band identified with the *upper arrow* in the *top panel* co-migrated with the EGF receptor. In addition to increasing the tyrosine phosphorylation of the p46 and p52 forms of SHC, small increases in the tyrosine phosphorylation of p66 SHC were observable at this exposure. For all stimuli except NGF, the treatment of cells with AG1478 reduced the association of Grb2 with SHC and blocked the tyrosine phosphorylation of SHC, the EGF receptor, and other proteins. The tyrosine-phosphorylated protein at the *lower arrow* in the *upper panel* was not identified.

Grb2 with SHC was also blocked by AG1478, except for that promoted by NGF (Fig. 6, *lower panel*). These results suggest that the tyrosine phosphorylation of SHC by UTP, ionomycin, and PMA is downstream of the activation of the EGF receptor. Consistent with this, these stimuli promoted an increase in the tyrosine phosphorylation of a protein that was co-immunoprecipitated with SHC and that co-migrated with the EGF receptor (Fig. 6, *upper panel*), and this was reduced in cells pretreated with AG1478. These results, along with the effects of AG1478 on MAP kinase activity (Fig. 4), suggest that the EGF receptor is in a signaling cascade that is upstream of MAP kinase and downstream of the activation of the P_{2Y2} receptor, the elevation of $[Ca^{2+}]_i$, and the production of DAG.

DISCUSSION

The results of these studies demonstrate that activation of the P_{2Y2} receptor by UTP stimulates MAP kinase in PC12 cells via a cascade of signaling proteins that include RAFTK and the EGF receptor. UTP, which increases $[Ca^{2+}]_i$ in these cells (23, 24),[2] promotes the tyrosine phosphorylation of RAFTK (Fig. 2A). This is reduced by down-regulating PKC (Fig. 2B) as well as by blocking the elevation of $[Ca^{2+}]_i$ (1), and both of these alterations reduce the UTP-promoted activation of MAP kinase (1). UTP, ionomycin, and PMA promote the tyrosine phosphorylation of the EGF receptor (Fig. 3). The effects of these stimuli on MAP kinase activity (Fig. 4), the tyrosine phosphorylation of SHC (Fig. 6), and the association of Grb2 with SHC (Fig. 6) were reduced by blocking the EGF receptor tyrosine kinase with the tyrphostin AG1478.

A model for the signaling cascade between the P_{2Y2} receptor and MAP kinase is shown in Fig. 7. In this model, RAFTK is upstream of the EGF receptor and downstream of the elevation of $[Ca^{2+}]_i$ and PKC, and the activation of both the EGF receptor and RAFTK is critical to the P_{2Y2} receptor-dependent stimulation of MAP kinase. The results are also consistent with the critical involvement of RAFTK and the EGF receptor in the

effects of both ionomycin and PMA on MAP kinase activation in PC12 cells. The EGF receptor may act as a scaffolding or adaptor protein to help to coordinate the signaling molecules involved in the MAP kinase activation scheme outlined in the model (Fig. 7). In this way, the EGF receptor and other proteins (reviewed in Ref. 14) may provide an organizational role beyond their intrinsic biochemical function. In this model, the active tyrosine kinase activity of the EGF receptor is also required, since AG1478 blocks the stimulation of MAP kinase and SHC tyrosine phosphorylation.

An increasing number of studies have demonstrated the involvement of the EGF receptor and various signaling proteins, including SHC and Src, in the activation of MAP (ERK) kinase by GPCRs. The EGF receptor can be activated by signaling events initiated by $G\beta\gamma$ subunit (5), ionomycin (19, 25), and agonists for lysophosphatidic acid, thrombin, endothelin 1, and angiotensin II receptors (5, 9–11). In neuronal cell types, including PC12 cells, $[Ca^{2+}]_i$ increases produced by GPCR ligands, calcium ionophores, or membrane depolarization, have been shown to promote neurite outgrowth and activate the EGF receptor, MAP kinase, and other signaling proteins (19, 20, 25, 26). Similar to results presented here, the tyrphostin AG1478 blocked MAP kinase activation and the tyrosine phosphorylation of SHC and other signaling proteins promoted by ligands to GPCRs in other studies (9–11, 19). Although there are a number of similarities in the signaling cascades downstream of the GPCRs in different cellular systems, there also are differences. GPCR- and $G\beta\gamma$-mediated tyrosine phosphorylation of the EGF receptor and SHC were also found to be dependent on Src kinase, and these events were upstream of MAP kinase activation (5). Since an autophosphorylation-specific antibody did not detect an increase in phosphorylation, these increases in MAP kinase activity did not appear to require the intrinsic kinase activity of the EGF receptor (5). This conclusion is different from that reached in the present study and one that used both AG1478 and the expression of dominant negative EGF receptor (10). It is therefore of interest that growth hormone, which binds to a receptor that is a member of the cytokine receptor family, promoted an increase in MAP kinase that was dependent on an increase in EGF receptor tyrosine phosphorylation, but the intrinsic tyrosine kinase activity of the EGF receptor was not required for the activation of MAP kinase (27). Thus, the EGF receptor plays an important role in MAP kinase activity in different cell types, but in some cases its kinase activity may not be required.

A number of studies indicate that Src is involved in mediating the activation of MAP kinase by GPCRs. Src can co-immunoprecipitate with the EGF receptor in cells exposed to ligands to GPCRs, including lysophosphatidic acid (5) and angiotensin II (11). In PC12 cells, the activation of GPCRs promoted the association of PYK2 (RAFTK) and activated Src, and this coupling was involved in the activation of MAP kinase (7). Through the use of dominant negative PYK2 and Src proteins, it was demonstrated that PYK2 and Src were involved in mediating the activation of MAP kinase by the G_i-coupled α2A-adrenergic receptor and the G_q-coupled α1B-adrenergic receptor at a point upstream of SHC in nonneuronal cells (8). An inhibitor (PP1) of Src-like kinases blocked the effects of both GPCR- and EGF receptor-promoted activation of MAP kinase and tyrosine phosphorylation of signaling proteins (including SHC) downstream of EGF receptor activation but produced only a modest reduction in the lysophosphatidic acid-promoted tyrosine phosphorylation of the EGF receptor (10). This suggests that Src or other kinases may play a regulatory role immediately distal to GPCR-mediated EGF receptor tyrosine kinase activation. A similar conclusion was reached concerning

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[2] S. P. Soltoff, unpublished results.

RAFTK and EGFR Mediate GPCR-promoted MAP Kinase Activation

PMA (acute)   UTP   Ionomycin   EGF

DAG ← PIP$_2$ PLC G P2Y2R   EGFR

PKC   IP$_3$

↑[Ca^{2+}]$_i$ ← AG1478

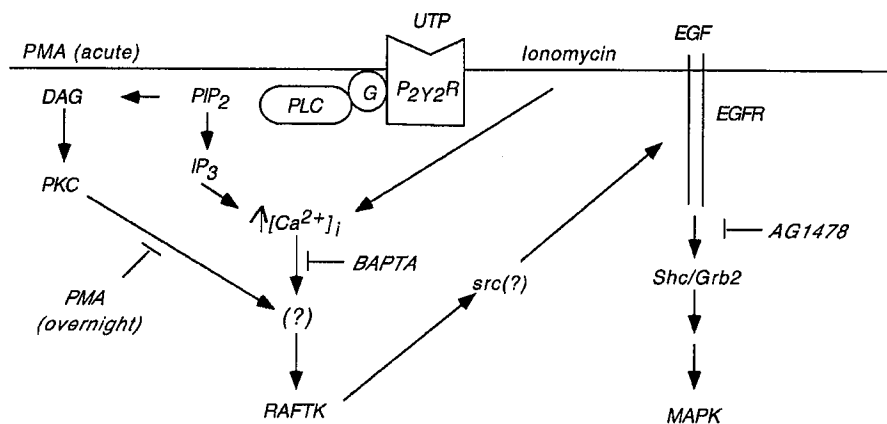PMA (overnight)   ← BAPTA   src(?)   Shc/Grb2

(?)

RAFTK   MAPK

Fig. 7. **Model for the transactivation of the EGF receptor and the stimulation of MAP kinase by UTP, phorbol ester, and [Ca^{2+}]$_i$ elevation.** UTP binds to P$_{2Y2}$ receptors and activates PLC, thereby promoting the hydrolysis of phosphatidylinositol 4,5-P$_2$ (PIP$_2$) to DAG and inositol-1,4,5-trisphosphate (IP$_3$). The resulting stimulation of PKC and the elevation of [Ca^{2+}]$_i$ activates RAFTK. PMA and ionomycin also activate RAFTK. Subsequently, the EGF receptor is activated, SHC is phosphorylated on tyrosine residues, and Grb2 is recruited to SHC. MAP kinase is activated by the signaling cascade downstream of SHC/Grb2. The activation of RAFTK is blocked by down-regulating PKC by treating cells overnight with PMA. RAFTK is also blocked by loading the cells with the Ca^{2+} chelator BAPTA-AM (1). The tyrphostin AG1478 blocks the tyrosine phosphorylation of the EGF receptor and all processes downstream of the activation of this receptor. Thus, AG1478 blocks the effects of UTP, ionomycin, and PMA on MAP kinase, SHC phosphorylation, and the activation of other signaling proteins. See "Discussion" for more details.

the involvement of Src and the activation of the PDGFα receptor by PDGF. Src was required for efficient PDGF-dependent tyrosine phosphorylation of SHC but not for tyrosine phosphorylation of the receptor itself or for other signaling proteins (phospholipase C-γ1, SHP2) that are recruited to this receptor (28).

The model for the P$_{2Y2}$ receptor-mediated activation of MAP kinase is generally compatible with many of the studies cited above. Src and/or Src-like kinases may play regulatory roles at multiple points in the signaling cascade between growth factor/G-protein-coupled receptors and MAP kinase, and this may vary with different kinds of cells or GPCRs. In addition, although it is not shown explicitly in the model, the UTP- and PMA-dependent activation of MAP kinase may also involve PKC-mediated effects that are independent of RAFTK and the EGF receptor, since PKC can increase MAP kinase by directly phosphorylating Raf-1 (29). Based on the degree of inhibition of MAP kinase activity by AG1478, the major P$_{2Y2}$-mediated pathway is that outlined in Fig. 7.

Several observations suggest that the acute activation of PKC by PMA can produce a positive effect on the EGF receptor, including (a) the PMA-promoted increase in the tyrosine phosphorylation of the EGF receptor (Figs. 3B and 6) and (b) the inhibition by AG1478 of the PMA-promoted MAP kinase activation (Fig. 4) and SHC tyrosine phosphorylation (Fig. 6). In other reports, topical treatment of mice with PMA increased the tyrosine phosphorylation of the EGF receptor in epidermal tissue (30). Of related interest, PMA and PKC activation also promoted the tyrosine phosphorylation and activation of ErbB2 and ErbB3, other members of the EGF receptor family, in a rat hepatoma cell line (31). However, many studies have demonstrated that PMA and ligands to PLC-linked GPCRs, including P$_2$ receptors (32), reduce the basal and/or EGF-dependent tyrosine kinase activity of the EGF receptor and reduce signaling by the EGF receptor (Ref. 33; for a review, see Ref. 34). Down-regulation of the EGF receptor tyrosine kinase activity may involve phosphorylation of Thr[654] and Thr[669] by PKC and MAP kinase, respectively, as well as the phosphorylation of additional sites (35, 36). Thus, it appears that the EGF receptor tyrosine kinase can be either activated (as in our studies) or inhibited by ligands to GPCRs and other stimuli.

One may speculate that Src and/or RAFTK, which can be activated by either an elevation of [Ca^{2+}]$_i$ or PMA, may con-

tribute to whether there is either GPCR-mediated activation or inhibition of the EGF receptor, but this will require a direct examination. Interestingly, ATP also produced an increase in MAP kinase activity in another PC12 cell line via the activation of an ionotropic P$_{2X}$-type receptor (which is not coupled to a G-protein) and not via a metabotropic P$_{2Y}$-type receptor (37). Although K$^+$-stimulated depolarization and ATP both produced extracellular Ca^{2+}-dependent increases in RAFTK (PYK2) tyrosine phosphorylation and MAP kinase activity in these cells, neither stimulus was found to increase the tyrosine phosphorylation of the EGF receptor.

NGF produced a small increase in the tyrosine phosphorylation of the EGF receptor in anti-EGF immunoprecipitates in some experiments (not shown) and in a protein (presumably the EGF receptor) that co-migrated with the EGF receptor in anti-SHC immunoprecipitates (Fig. 6). This is likely to reflect changes in the tyrosine phosphorylation of the EGF receptor by the elevation in [Ca^{2+}]$_i$ or the production of DAG in NGF-treated cells. The lack of effect of a significant inhibitory effect of AG1478 on the NGF-promoted tyrosine phosphorylation of SHC (Fig. 6), association of Grb2 with SHC (Fig. 6), and the activation of MAP kinase (not shown) are consistent with the p140^{trk}-mediated activation of MAP kinase by NGF (38). However, the effects of NGF on the EGF receptor tyrosine phosphorylation suggest that there is cross-talk between these two growth factor receptors as well as between GPCRs and growth factor receptors. Transmodulation of the EGF receptor by the PDGF receptor has been reported (reviewed in Ref. 39). In addition, recent studies indicate that the EGF receptor and the PDGFβ receptor can interact directly with each other, perhaps by heterodimerization or oligomerization (40).

P$_{2Y2}$ receptors are present on many different types of tissues and cells in culture (reviewed in Refs. 2–4). These receptors may play a role in autocrine or paracrine signaling due to the release of intracellular ATP by physiological and pathophysiological conditions. Platelet secretory granules contain high concentrations of ADP and ATP, and platelets have P$_2$ receptors. ATP is co-stored and co-released with neurotransmitters and can act as a neurotransmitter itself (42–44). P$_2$ receptor-mediated effects of extracellular nucleotides on neuronal cells include MAP kinase activation, the formation of AP-1 complexes, and trophic effects (45). Cytosolic ATP may be lost from some epithelial cells by efflux through the CFTR channel, which may

act as both a chloride channel and ATP channel (46, 47). In this role, cytosolic ATP has been suggested to leave the cells and subsequently activate P_{2Y2} receptors and thereby activate other chloride channels.

MAP kinase is activated by many growth factors and by various GPCRs in different cells. The effects of UTP and EGF on signaling molecules that are upstream of MAP kinase are representative of both similarities and differences in GPCR-mediated and receptor tyrosine kinase-mediated signaling in PC12 cells. Both stimuli, as well as ionomycin and PMA, increased the tyrosine phosphorylation of the EGF receptor and SHC. The transactivation of the EGF receptor was required for the full activation of MAP kinase by the P_{2Y2} receptor-mediated stimulus as well as for MAP kinase activation promoted by ionomycin and PMA. As such, these studies illustrate the interactions that may occur between different types of receptors and demonstrate how Ca^{2+} and DAG can initiate signaling cascades that reproduce the downstream effects of GPCR-mediated signaling.

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Calcium-dependent Epidermal Growth Factor Receptor Transactivation Mediates the Angiotensin II-induced Mitogen-activated Protein Kinase Activation in Vascular Smooth Muscle Cells*

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We have recently reported that angiotensin II (Ang II)-induced mitogen-activated protein kinase (MAPK) activation is mainly mediated by Ca^{2+}-dependent activation of a protein tyrosine kinase through G_q-coupled Ang II type 1 receptor in cultured rat vascular smooth muscle cells (VSMC). In the present study, we found Ang II rapidly induced the tyrosine phosphorylation of the epidermal growth factor (EGF) receptor and its association with Shc and Grb2. These reactions were inhibited by the EGF receptor kinase inhibitor, AG1478. The Ang II-induced phosphorylation of the EGF receptor was mimicked by a Ca^{2+} ionophore and completely inhibited by an intracellular Ca^{2+} chelator. Thus, AG1478 abolished the MAPK activation induced by Ang II, a Ca^{2+} ionophore as well as EGF but not by a phorbol ester or platelet-derived growth factor-BB in the VSMC. Moreover, Ang II induced association of EGF receptor with catalytically active c-Src. This reaction was not affected by AG1478. These data indicate that Ang II induces Ca^{2+}-dependent transactivation of the EGF receptor which serves as a scaffold for pre-activated c-Src and for downstream adaptors, leading to MAPK activation in VSMC.

Protein tyrosine phosphorylation and subsequent protein-protein interaction induced by growth factors is a prototypical pathway to transmit mitogenic signals to the nucleus (1). For example, tyrosine phosphorylation of growth factor receptors recruits the guanine nucleotide exchange factor, son-of-sevenless (Sos)[1] through adaptor proteins, Shc and Grb2, thereby initiating a sequential cascade from p21^{ras} (Ras) to mitogen-activated protein kinases (MAPKs), referred to as p44^{mapk}

(ERK1) and p42^{mapk} (ERK2) (2–5). MAPKs in turn phosphorylate and activate several kinases and transcriptional factors, including TCF/Elk1, and stimulate the induction of c-fos (4, 6).

Angiotensin II (Ang II), a major effector peptide of the renin-angiotensin system, is now believed to play a critical role in the pathogenesis of cardiovascular remodeling associated with hypertension, heart failure, and atherosclerosis (7). We and others (8, 9) have previously cloned the Ang II type 1 receptor (AT$_1$) which not only mediates diverse hemodynamic effects of Ang II (10) but also promotes hypertrophy and/or hyperplasia of vascular smooth muscle cells (VSMC) (11–13), cardiomyocytes (14), and cardiac fibroblasts (15). AT$_1$ belongs to the superfamily of heterotrimeric G protein-coupled receptors (GPCR) (8, 9). In cultured VSMC, AT$_1$ activates phospholipase C (PLC), which initiates the generation of inositol trisphosphate and diacylglycerol, causing intracellular calcium mobilization and protein kinase C activation, respectively (16, 17). In addition, Ang II induces several signaling events commonly evoked by growth factor receptors, such as the activation of MAPK (18, 19) and the ribosomal S6 kinase (20), and the expression of the nuclear proto-oncogenes, c-fos, c-jun, and c-myc (21–23) in VSMC.

Although AT$_1$ lacks intrinsic tyrosine kinase activity, it appeared to induce tyrosine phosphorylation of multiple signaling proteins (24) including Shc (25), focal adhesion kinase (26), paxillin (27), PLC-γ (28), JAK2, and STAT1 (29) in VSMC, suggesting cross-talk of AT$_1$ and a tyrosine kinase. In fact, recent works with various GPCR including AT$_1$ suggest that GPCR-induced MAPK activation requires Shc-Grb2·Sos and/or Grb2·Sos complex formation and subsequent Ras activation mediated by several candidate tyrosine kinases, such as proline-rich tyrosine kinase 2 (PYK2) (30), platelet-derived growth factor (PDGF) receptor (25), epidermal growth factor (EGF) receptor (31), and Src family tyrosine kinases (32–35).

We have recently reported that Ang II-induced Ras and MAPK activation is mainly mediated by a calcium-dependent protein tyrosine kinase through G_q-mediated PLC activation via AT$_1$ in cultured rat quiescent VSMC (36). However, the identity of the tyrosine kinase and its pathophysiological significance in the growth promoting signal of Ang II have remained unclear. In the present study, we found that Ang II induces Ca^{2+}-dependent tyrosine phosphorylation of the EGF receptor to recruit Shc and Grb2, thereby activating MAPK in VSMC. The transactivation of the EGF receptor seems to be an essential point of convergence in this growth promoting cascade because it provides docking sites for the upstream tyro-

[1] The abbreviations used are: Sos, son-of-sevenless; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; Ang II, angiotensin II; AT$_1$, angiotensin II type 1 receptor; VSMC, vascular smooth muscle cells; GPCR, G protein-coupled receptor; PLC, phospholipase C; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; mAb, monoclonal antibody; pAb, polyclonal antibody.

sine kinase c-Src and downstream adaptors at the plasma membrane, and because its activity is required for the MAPK activation induced by Ang II.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Chemicals and reagents were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, penicillin, and streptomycin from Life Technologies, Inc.; Ang II from Peninsula Laboratories; recombinant human EGF and PDGF-BB from Upstate Biotechnology Inc.; AG1478, AG1295, A23187, and BAPTA-AM from Calbiochem; phorbol 12-myristate 13-acetate and EGTA from Sigma; an agarose-conjugated glutathione *S*-transferase (GST)-Grb2-(1–217) fusion protein and protein A/G-agarose from Santa Cruz Biotechnology. CV11974 was a generous gift of Takeda Pharmaceutical Co.

Antibodies and Their Specificities—The rabbit polyclonal phospho-specific MAPK antibody (9101) raised against a synthetic phosphotyrosine peptide corresponding to amino acids 196–209 (DHTGFLTEY-(*P*)VATRWC, where *P* indicates phosphate) of human p44 MAPK (ERK1) was obtained from New England Biolabs that detects only the catalytically active form of p42/44 MAPKs which are phosphorylated at Tyr^{204}. We have previously shown (36) that this antibody specifically recognizes Tyr^{204}-phosphorylated p42/44 MAPK in cultured rat VSMC. Anti-EGF receptor polyclonal antibody (pAb)(1005) raised against a synthetic peptide corresponding to amino acid residues 1005–1016 of human EGF receptor (identical to the corresponding mouse sequence) was obtained from Santa Cruz Biotechnology that also specifically recognizes rat EGF receptor in both immunoblotting and immunoprecipitation. Anti-Shc pAb (06-203) and monoclonal antibody (mAb) (S52420: clone 8) raised against a GST-tagged fusion protein corresponding to the SH2 domain (amino acid residues 366–473) of the human p46/p52 Shc was obtained from Upstate Biotechnology and Transduction Laboratories, respectively. Anti-Shc pAb specifically reacts with p46/52/66 Shc of rat origin by immunoblotting and immunoprecipitation (34, 37), and anti-Shc mAb also reacts with p46/52/66 Shc of rat origin by immunoblotting (34, 38). Anti-Grb2 pAb (C-23) raised against a peptide corresponding to amino acid residues 195–217 of human Grb2 was obtained from Santa Cruz Biotechnology that is also specific for Grb2 of the rat origin by immunoprecipitation (34, 38). Anti-Grb2 mAb (G16720: clone 24) raised against the entire 24-kDa Grb2 protein from rat brain was obtained from Transduction Laboratories that specifically reacts with rat Grb2 by immunoblotting (30). Anti-Sos pAbs (S15530) raised against a protein fragment of mouse Sos1 corresponding to amino acid residues 1–109 and a protein fragment of mouse Sos2 corresponding to amino acid residues 1095–1297, respectively, were obtained from Transduction Laboratories that also specifically react with Sos1 and Sos2 of rat origin by immunoblotting (34). Anti-PDGF β receptor pAb (06-498) raised against a synthetic peptide corresponding to amino acid residues 1013–1025 of human PDGF β receptor was obtained from Upstate Biotechnology. It specifically reacts with PDGF β receptor by immunoblotting and immunoprecipitation. We have previously confirmed that this antibody also specifically reacts with rat PDGF β receptor in cultured rat VSMC (39). The mAb directed to Tyr^{530}-dephosphorylated c-Src (clone 28) was prepared as described previously which selectively recognizes the active form of rat c-Src (40). A horseradish peroxidase-conjugated recombinant antibody fragment specific for phosphotyrosine (RC20) and anti-phosphotyrosine mAb (4G10) were from Transduction Laboratories and Upstate Biotechnology Inc., respectively. Horseradish peroxidase-conjugated second antibodies were from Amersham Pharmacia Biotech.

The specificities of the antibodies used in the present study have been described in publications as referred to above as well as elsewhere. In addition, the rabbit pAbs used in the present immunoprecipitation studies (1005, 06-203, C-23), except control normal rabbit IgG or the pAb preincubated with its immunogen, specifically immunoprecipitated target proteins that, upon SDS-polyacrylamide gel electrophoresis, migrated to positions calculated from their molecular weights when visualized by the respective pAbs (1005) or mAbs (S52420, G16720) used in the present immunoblotting studies (Fig. 4C and data not shown), thus confirming that the precipitated proteins are specific to the precipitating antibodies and that the bands detected by the blotting antibodies are specifically recognized by the antibodies.

Cell Culture—VSMC were prepared from the thoracic aorta of 12-week-old Sprague-Dawley rats (Charles River Breeding Laboratories) by the explant method and cultured in DMEM containing 10% fetal calf serum, penicillin, and streptomycin as described previously (41). Subcultured VSMC from passages 3–15, used in the experiments, showed >99% positive immunostaining of smooth muscle α-actin antibody and were negative for mycoplasma infection. The expression of AT$_1$ but not AT$_2$ receptors was confirmed on the basis of binding studies with specific receptor antagonists (36). Cells at ~80% confluence in culture wells were made quiescent by incubation with serum-free DMEM for 3 days.

MAPK Activity—VSMC grown on a 24-well plate were stimulated with agonists at 37 °C in serum-free DMEM for specified durations. The reaction was terminated by the replacement of medium with the ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 20 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na$_3$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After brief sonication (10 s), the samples were centrifuged for 5 min at 14,000 × g, and the supernatant was assayed for MAPK activity with an assay kit (Amersham Pharmacia Biotech) that measures the incorporation of [^{33}P]phosphate from [$γ$-^{33}P]ATP into a synthetic peptide (KRELVEPLTPAGEAP-NQALLR) as a specific MAPK substrate (36). The reaction was carried out with the cell lysate (~1 μg of protein) in 75 mM HEPES buffer, pH 7.4, containing 1.2 mM MgCl$_2$, 2 mM substrate peptide, and 1.2 mM ATP, 1 μCi of [$γ$-^{33}P]ATP for 30 min at 30 °C. The resultant solution was applied to a phosphocellulose membrane and extensively washed in 1% acetic acid and then in deionized water. The radioactivity trapped on the membrane was measured by liquid scintillation counting.

Immunoprecipitation and Immunoblotting—Cells were lysed by adding ice-cold lysis buffer, pH 7.5, containing 50 mM HEPES, 50 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl$_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 1 mM Na$_3$VO$_4$, 100 mM NaF, 30 mM 2-(*p*-nitrophenyl) phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin and centrifuged for 5 min at 14,000 × g. Supernatant was mixed with the immunoprecipitation antibody and rocked at 4 °C for 2–16 h, and then protein A/G-Sepharose was added for an additional 2 h to overnight. Immunoprecipitates were washed 3 times in the lysis buffer, solubilized in Laemmli sample buffer with 2-mercaptoethanol, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). After blocking with 5% milk, the membrane was treated with a primary antibody followed by a secondary antibody conjugated with horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). For repeated immunoblotting, membranes were stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 0.1 M 2-mercaptoethanol for 30–45 min at 50 °C. For immunoblot analysis of MAPK phosphorylation, VSMC grown on a 6-well plate were directly lysed by Laemmli sample buffer with 2-mercaptoethanol, resolved by SDS-polyacrylamide gel electrophoresis, and subjected to immunoblotting. For immunoblot analysis of Grb2-associated proteins, agarose-conjugated GST-Grb2 fusion protein was rocked with Triton X-100 lysate of VSMC for 2 h to overnight at 4 °C and washed 3 times with lysis buffer. Bound proteins were solubilized, resolved by SDS-polyacrylamide gel electrophoresis, and subjected to immunoblotting, as described above.

Reproducibility of the Results—Unless stated otherwise, results are representative of at least three experiments giving similar results.

RESULTS

Inhibition of Ang II-induced MAPK Activation by AG1478, the EGF Receptor Kinase Inhibitor—In many MAPK activation systems, a tyrosine-phosphorylated scaffold is needed to assemble adaptor proteins (1–3). Highly likely candidates in VSMC for such a scaffold mediating MAPK activation by Ang II are the PDGF or EGF receptors. In VSMC, Ang II has been shown to cause PDGF β receptor phosphorylation which leads to recruitment of the Shc·Grb2 complex to the receptor (25). This pathway may account for Ras and MAPK activation. A recent study revealed that several GPCRs use EGF receptor transactivation for MAPK activation, c-*fos* induction, and DNA synthesis in Rat-1 fibroblasts (31). To clarify the role of these receptor tyrosine kinases in Ang II-induced signal transduction in VSMC, we first tested the effect of selective receptor tyrosine kinase inhibitors (42) on the MAPK activity in VSMC. As shown in Fig. 1A, the EGF receptor kinase inhibitor tyrphostin AG1478 dose-dependently and completely blocked EGF-induced MAPK activation, whereas it had no effect on the PDGF-induced activation confirming its stringent selectivity. We then observed marked inhibition of Ang II-induced MAPK activation
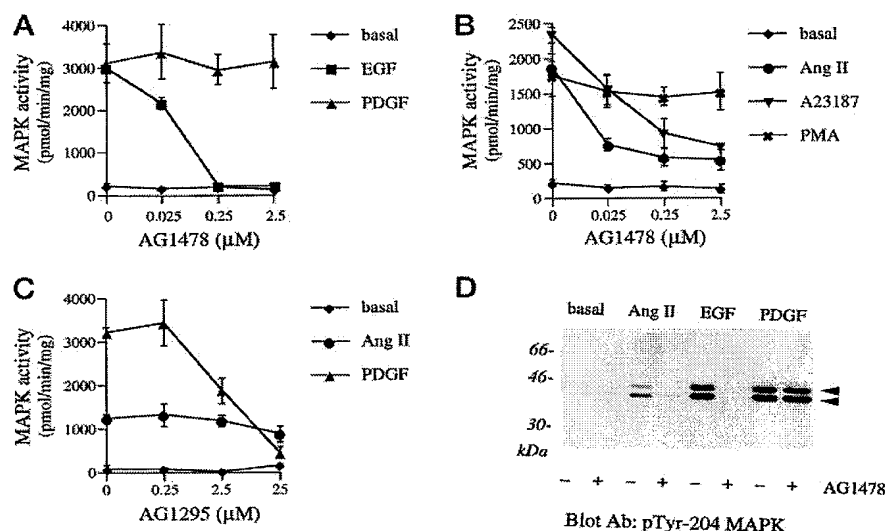
FIG. 1. **Inhibition of Ang II-induced MAPK activation by the EGF receptor kinase inhibitor AG1478.** *A* and *B*, VSMC were pretreated with indicated concentrations of AG1478 for 30 min and stimulated with EGF (100 ng/ml) or PDGF-BB (100 ng/ml) (*A*) and Ang II (100 nM), A23187 (10 μM), or phorbol 12-myristate 13-acetate (*PMA*) (1 μM) (*B*) for 5 min. The MAPK activity of cell lysate was determined as described under "Experimental Procedures." Results shown are mean ± S.D. of at least triplicate determinations. *C*, VSMC were pretreated with indicated concentrations of the PDGF receptor kinase inhibitor AG1295 for 30 min and stimulated with Ang II (100 nM) for 5 min. Results shown are mean ± S.D. of at least triplicate determinations. *D*, VSMC were pretreated with or without 250 nM AG1478 for 30 min and stimulated with Ang II (100 nM), EGF (100 ng/ml), or PDGF-BB (100 ng/ml) for 10 min. Immunoblotting with antibody specific for phosphorylated MAPK was performed as described under "Experimental Procedures." *Arrows* indicate tyrosine-phosphorylated p44^{mapk} and p42^{mapk}.

by AG1478 (Fig. 1*B*) but not by the PDGF receptor-selective tyrosine kinase inhibitor, AG1295 (Fig. 1*C*). AG1478 also inhibited the tyrosine phosphorylation of MAPK by Ang II and EGF without affecting that by PDGF-BB (Fig. 1*D*). Moreover, AG1478 inhibited Ca^{2+} ionophore (A23187)-induced MAPK activation, whereas it had no effect on the phorbol ester-induced activation (Fig. 1*C*). This is in good agreement with our previous observation that the Ang II-induced Ras and MAPK activation requires a Ca^{2+}-sensitive tyrosine kinase but not protein kinase C in VSMC (36).

Ang II Induces Calcium-dependent Tyrosine Phosphorylation of the EGF Receptor and Its Association with Shc, Grb2, and Sos—The activated EGF receptor can recruit the Grb2·Sos complex directly and indirectly via tyrosine phosphorylation of Shc, thereby activating the Ras/MAPK signaling pathway (1–3). To examine the possibility that Ang II signaling utilizes the EGF receptor to provide a docking site for Grb2, we examined proteins that interact with a GST-Grb2 fusion protein in the lysate of VSMC upon stimulation by Ang II. As shown in Fig. 2*A*, Ang II transiently increased association of several tyrosine-phosphorylated proteins with the GST-Grb2 fusion protein as detected by anti-phosphotyrosine antibody. The association of tyrosine-phosphorylated proteins is specific to Grb2 because no band was seen when GST-agarose alone was used (Fig. 2*D*). A similar pattern of Grb2-associating tyrosine-phosphorylated proteins was observed following treatment of VSMC with the Ca^{2+} ionophore, A23187 (Fig. 2*B*). The major phosphoprotein (~170 kDa) associated with the GST-Grb2 fusion protein upon treatment with Ang II was identified as the EGF receptor because it was recognized by the anti-EGF receptor antibody (Fig. 2*C*) and was diminished by pretreatment with AG1478 (data not shown). We confirmed that the phosphorylated EGF receptor was coprecipitated with endogenous Grb2 upon Ang II treatment and was also diminished by AG1478 (Fig. 3). It should be noted that the tyrosine-phosphorylated bands of ~50 kDa were practically wiped out by AG1478, whereas the ~120-kDa band was not visibly affected (Fig. 3). In cultured rat VSMC, Ang II was shown to induce tyrosine phosphorylation of

46, 52, and 66 kDa Shc isoforms which subsequently form a complex with Grb2 (25). The ~50-kDa phosphoprotein associated with Grb2 upon treatment with Ang II shown in Fig. 3 should be p52 Shc. Indeed, the Ang II treatment resulted in association of 46-, 52-, and 66-kDa Shc isoforms to the GST-Grb2 fusion protein in which p52 Shc was the dominant form in VSMC (Fig. 2*A*). A23187 also increased p52 Shc association to the GST-Grb2 fusion protein in VSMC (Fig. 2*B*).

Since Shc and Grb2 specifically recognize tyrosine-phosphorylated proteins (1–3), the Ang II-induced association of the EGF receptor and Shc with the GST-Grb2 fusion protein suggests that Ang II causes tyrosine phosphorylation of the EGF receptor. To confirm the involvement of the EGF receptor and to examine the effect of Ang II on the phosphotyrosine content of the EGF receptor, it was immunoprecipitated and analyzed by immunoblotting with an anti-phosphotyrosine antibody. As shown in Fig. 4*A*, Ang II induced tyrosine phosphorylation of the EGF receptor within 1 min, peaked at 2 min, and declined in 5 min. We further confirmed that the band recognized by anti-phosphotyrosine antibody specifically represents the tyrosine-phosphorylated EGF receptor because neither phosphorylated band nor immunoprecipitated EGF receptor could be observed when immunoprecipitation antibody (anti-EGF receptor antibody) was preabsorbed with its immunogen (Fig. 4*C*). Moreover, Ang II increased the amount of Shc, Grb2, and Sos which was coprecipitated with the EGF receptor (Fig. 4*A*). The phosphorylation of the EGF receptor was also observed upon treatment of VSMC with the Ca^{2+} ionophore A23187 (Fig. 4*B*). In good agreement with our previous observation that Ang II-induced Ras and MAPK activation is mainly mediated through intracellular Ca^{2+} mobilization (36), the Ang II-induced phosphorylation of the EGF receptor was completely inhibited by an intracellular Ca^{2+} chelator, BAPTA-AM, but only partially affected by an extracellular Ca^{2+} chelator, EGTA (Fig. 4*C*).

Ang II has been shown to stimulate the secretion of several growth factors in cultured VSMC, some of which have not been identified yet (13). It is possible that the Ang II-induced EGF
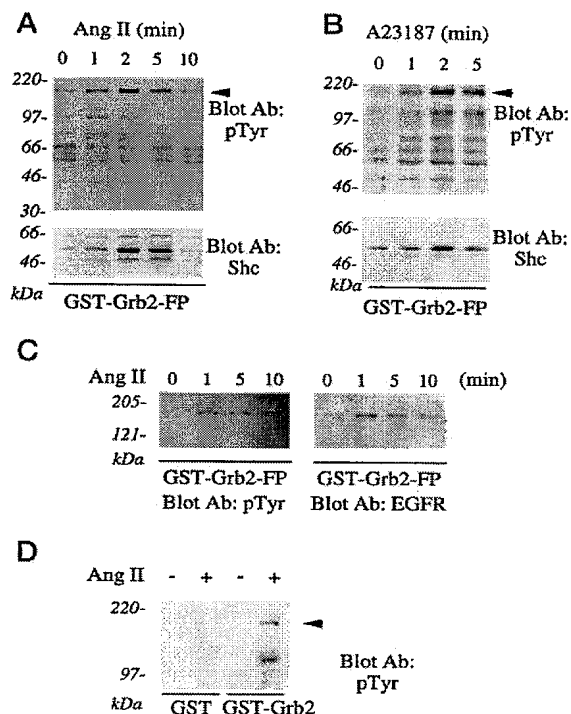
FIG. 3. **Tyrosine-phosphorylated EGF receptor is coprecipitated with endogenous Grb2 upon Ang II treatment.** VSMC were pretreated with or without AG1478 (250 nM) for 30 min and stimulated with Ang II (100 nM) for indicated durations. After cell lysis, immunoprecipitation (*IP*) was performed with anti-Grb2 pAb. Precipitates were analyzed by repeated immunoblotting with anti-phosphotyrosine mAb, anti-EGF receptor pAb, and anti-Grb2 mAb as indicated. *Arrowheads* indicate the 170-kDa tyrosine-phosphorylated EGF receptor and ~120- and ~50-kDa phosphorylated proteins, respectively. *DMSO*, dimethyl sulfoxide; *pTyr*, phosphotyrosine; *EGFR*, EGF receptor.

FIG. 2. **Ang II and a Ca^{2+} ionophore induced association of tyrosine-phosphorylated EGF receptor and Shc to GST-Grb2 fusion protein.** *A–C*, VSMC were stimulated with Ang II (100 nM) (*A* and *C*) or A23187 (10 μM) (*B*) for indicated durations. After cell lysis, GST-Grb2 fusion protein immobilized on glutathione-agarose beads was added. Proteins associated with the fusion protein were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine mAb, anti-Shc pAb, or anti-EGF receptor (*EGFR*) pAb as indicated. *Arrows* indicate the 170-kDa tyrosine-phosphorylated protein. *D*, VSMC were stimulated with or without Ang II (100 nM) for 2 min. After cell lysis, GST-Grb2 fusion protein, or GST alone, immobilized on glutathione-agarose beads was added. The associated proteins were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine mAb. *Arrowheads* indicate the 170-kDa tyrosine-phosphorylated protein. *DMSO*, dimethyl sulfoxide; *pTyr*, phosphotyrosine.

receptor activation occurs secondarily to autocrine secretion of EGF receptor ligands such as EGF or transforming growth factor-α. Since there are no reliable antibodies to rapidly neutralize multiple endogenous rat EGF receptor ligands (note that the transactivation takes place within 1 min) or to completely neutralize rat EGF receptor, we have excluded this possibility by examining the effect of the Ang II-treated conditioned medium on EGF receptor phosphorylation. As shown in Fig. 4*D*, the ability of the conditioned medium to phosphorylate the EGF receptor was lost when VSMC was pretreated with the AT$_1$ receptor antagonist, CV11974. Although there exists a small degree of basal phosphorylation of the PDGF β receptor, we could not detect further phosphorylation of the PDGF β receptor by Ang II during the time course in which we observed its EGF receptor phosphorylation (1~5 min) or its MAPK activation (2~10 min) in our VSMC (Fig. 5). These data indicate that the Ang II-induced EGF receptor transactivation, presumably through the intracellular Ca^{2+} elevation coupled to the AT$_1$ receptor, may account for the induced association of the phosphorylated receptor with Shc, Grb2, and Sos, and resultant Ras and MAPK activation.

Interaction of c-Src with Shc and EGF Receptor upon Ang II Stimulation—The Src family tyrosine kinases have been implicated in the GPCR-induced MAPK activation (32–35). c-Src
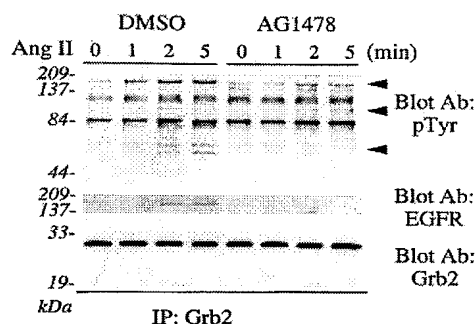
mediates Shc phosphorylation, Shc-Grb2 complex formation, and ensuing MAPK activation elicited by G$_i$-coupled receptors in COS-7 cells (35). In VSMC, Ang II was shown to activate c-Src (43) which may be required for the Ras activation by Ang II (44). Ca^{2+}-dependent c-Src activation was also reported in epidermal keratinocytes (45) and neuronal cells (46). To examine whether c-Src is involved in the MAPK cascade initiated by Ang II, the proteins inducibly associated with the GST-Grb2 fusion protein were immunoblotted by a monoclonal antibody, clone 28, which selectively recognizes the active (Tyr^{530}-dephosphorylated) form of c-Src (40). Ang II increased transient association of active c-Src with the GST-Grb2 fusion protein (Fig. 6*A*). Since Shc is known to be tyrosine-phosphorylated by Src kinases (47) presumably through the interaction with the SH3 domain of the kinases (48), we examined whether active c-Src forms a coprecipitable complex with Shc in response to Ang II. As shown in Fig. 6*B*, Ang II induced complex formation of active c-Src with Shc that was correlated with p52 Shc tyrosine phosphorylation in VSMC.

Recent studies indicate that c-Src is required for mitogenic effects of EGF (49). In human breast carcinoma cell lines, c-Src has been shown to be associated with the activated EGF receptor through its SH2 domain (50). It has been suggested that catalytically active c-Src phosphorylates the non-autophosphorylation site of the EGF receptor, Tyr^{891}, which serves as a binding site for c-Src (51). Therefore, we further tested the possibility that c-Src interacts with the EGF receptor upon stimulation by Ang II in VSMC. As shown in Fig. 6*C*, Ang II enhanced the association of active c-Src with the EGF receptor within 30 s. A23187 also increased the association (data not shown). The Ang II-induced association of active c-Src with the EGF receptor was still observable even in the presence of AG1478 (Fig. 6*C*). These data further suggest that Ang II could utilize c-Src to phosphorylate and associate with the EGF receptor, leading to recruitment of the downstream adaptors, Shc and Grb2, at the plasma membrane.

DISCUSSION

VSMC in culture has proven to be a useful model to examine the molecular mechanisms whereby vasoactive substances such as Ang II contribute to abnormal vascular hypertrophy. Recently, we have reported (36) that in VSMC, Ras and MAPK activation through AT$_1$ was mediated by a tyrosine kinase which may respond to G$_q$-coupled intracellular Ca^{2+} mobilization but not to protein kinase C activation. In the present
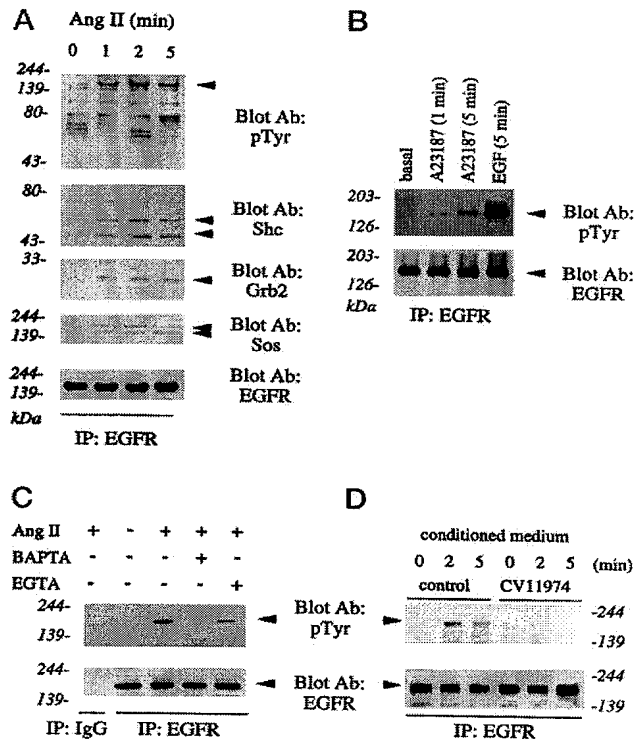
FIG. 4. Ang II stimulates tyrosine phosphorylation of EGF receptor through AT$_1$ receptor-mediated intracellular Ca^{2+} mobilization but not through autocrine release of the EGF receptor ligand. *A*, VSMC were stimulated with Ang II (100 nM) for indicated durations. After cell lysis, immunoprecipitation (*IP*) was performed with anti-EGF receptor pAb. Precipitates were analyzed by immunoblotting with anti-phosphotyrosine (*pTyr*) mAb, anti-Shc mAb, anti-Grb2 mAb, anti-Sos pAb, and anti-EGF receptor pAb by repeated reprobing. *Arrowheads* indicate the positions of 170-kDa tyrosine-phosphorylated EGF receptor, p46 and p52 Shc, Grb2, Sos1 and Sos2, PYK2, and EGF receptor (*EGFR*), respectively. *B*, VSMC were stimulated with A23187 (10 μM) or EGF (10 ng/ml) for indicated durations. After cell lysis, immunoprecipitation was performed with anti-EGF receptor pAb. Precipitates were analyzed by immunoblotting with anti-phosphotyrosine mAb and anti-EGF receptor pAb as indicated. *Arrowheads* indicate the position of EGF receptor. *C*, VSMC were pretreated with or without 10 μM BAPTA-AM or 5 mM EGTA and then stimulated with Ang II (100 nM) for 2 min. After cell lysis, immunoprecipitation was performed with anti-EGF receptor pAb or the anti-EGF receptor pAb preabsorbed with its immunogen (10 ×) for 2 h at 4 °C as a control. Precipitates were analyzed by immunoblotting with anti-phosphotyrosine mAb and anti-EGF receptor pAb. *Arrowheads* indicate the position of EGF receptor. *D*, VSMC were pretreated with or without the AT$_1$ receptor antagonist CV11974 (1 μM) for 30 min and then stimulated with conditioned medium from VSMC treated with Ang II (100 nM) for 2 min. After cell lysis, immunoprecipitation was performed with anti-EGF receptor pAb or the anti-EGF receptor pAb. Precipitates were analyzed by immunoblotting with anti-phosphotyrosine mAb and anti-EGF receptor pAb. *Arrowheads* indicate the position of EGF receptor.
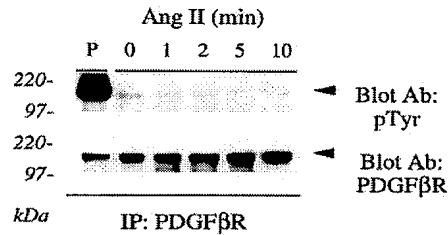
FIG. 5. Ang II does not affect phosphotyrosine content of PDGF β receptor. VSMC were stimulated with PDGF-BB (10 ng/ml) (*P*) for 5 min or Ang II (100 nM) for indicated durations. After cell lysis, immunoprecipitation (*IP*) was performed with anti-PDGF β receptor pAb. Precipitates were analyzed by immunoblotting with anti-phosphotyrosine (*pTyr*) mAb and anti-PDGF β receptor (*PDGFβR*) pAb. *Arrowheads* indicate the position of PDGF β receptor.

study, we have demonstrated that an EGF receptor kinase inhibitor, AG1478, selectively inhibited MAPK activation induced by Ang II and the Ca^{2+} ionophore A23187, whereas it had no effect on the activation induced by a phorbol ester. Furthermore, Ang II and A23187 induced tyrosine phosphorylation of the EGF receptor, which was sufficient to recruit the adaptor proteins that are involved in Ras activation. Thus, it is reasonable to speculate that the Ca^{2+}-dependent tyrosine phosphorylation of the EGF receptor may be a common mechanism to activate MAPK shared by several GPCRs coupled to G$_q$, such as AT$_1$ in VSMC. This notion is supported by the recent findings that several GPCR agonists (31) as well as

KCl-induced depolarization (38) elicited tyrosine phosphorylation of the EGF receptor and subsequent recruitment of the adaptor proteins to the receptor. In general, phosphorylation of the EGF receptor by GPCR agonists and Ca^{2+} agonists is relatively weaker than EGF itself as observed by us and others (31, 38) indicating that there may exist a threshold phosphorylation level of the receptor that is sufficient for the recruitment of the adaptors and subsequent MAPK activation.

AT$_1$ has been shown to mobilize intracellular Ca^{2+} by activating PLC-β through G$_q$ in cultured VSMC (16, 17). However, recent reports by Marrero *et al.* (28, 52) indicate that Ang II can mobilize intracellular Ca^{2+} by PLC-γ activation mediated by Src family tyrosine kinases in cultured rat VSMC. Since the EGF receptor can recruit and activate PLC-γ through an autophosphorylation site at Tyr^{992} (2), Ang II could elevate intracellular Ca^{2+} through PLC-γ activated by the EGF receptor thereby activating the MAPK cascade in VSMC. However, AG1478 (250 nM) did not influence Ang II-induced intracellular Ca^{2+} mobilization in VSMC.[2] We also showed that intracellular but not extracellular Ca^{2+} chelation was sufficient to inhibit EGF receptor phosphorylation induced by Ang II in the present study. Thus, the EGF receptor should be functionally downstream of the intracellular Ca^{2+} mobilization in the Ras-MAPK signal cascade originating at AT$_1$.

Although AG1478 is highly selective for the EGF receptor over other receptor tyrosine kinases (42), it is still possible that it affects other non-receptor kinases or signaling intermediates nonspecifically. This possibility is suspected by the fact that a higher dose of AG1478 was required for the inhibition of the MAPK activation by EGF than by Ang II. A similar phenomenon was also observed in Rat-1 fibroblasts which was attributed to the relatively weaker receptor phosphorylation by GPCR agonists than by EGF itself (31). In addition, pretreatment of VSMC with AG1478 tended to increase the amount of precipitated EGF receptor (see Fig. 6C). This may be due to the inhibition of basal level ubiquitination and subsequent proteolytic degradation of the receptor which requires the receptor tyrosine kinase activity (53). On the contrary, we found AG1478 had no effect on Ang II-induced association of c-Src to the EGF receptor nor on phorbol ester- or PDGF-BB-induced MAPK activation in the present study. Daub *et al.* (31) have reported that AG1478 inhibited the MAPK activation, c-*fos* mRNA expression, and DNA synthesis induced by endothelin-1 and thrombin, but it did not affect the tyrosine phosphorylation of focal adhesion kinase and paxillin by these agonists in Rat-1 fibroblasts. They have further demonstrated that these GPCR agonists failed to stimulate MAPK when Rat-1 cells were transfected with the dominant negative EGF receptor mutant. We
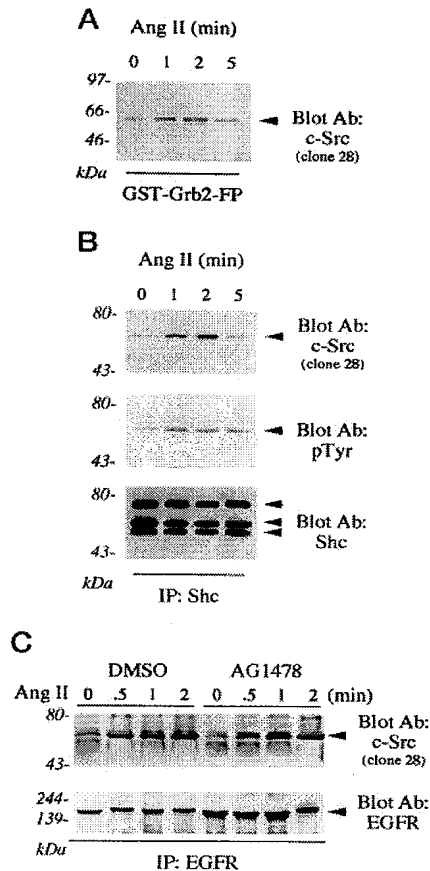
[2] S. Eguchi, unpublished data.

FIG. 6. **Interaction of c-Src with Shc, Grb2, and EGF receptor upon Ang II stimulation.** *A*, VSMC were stimulated with Ang II (100 nM) for indicated durations. After cell lysis, GST-Grb2 fusion protein immobilized on glutathione-agarose beads was added. Proteins associated with the fusion protein were separated by SDS-PAGE and immunoblotted with anti-c-Src mAb (clone 28) which selectively recognizes the catalytically active form of c-Src. *B*, VSMC were stimulated with Ang II (100 nM) for indicated durations. After cell lysis, immunoprecipitation (*IP*) was performed with anti-Shc pAb. Precipitates were analyzed by immunoblotting with anti-c-Src mAb (clone 28), anti-phosphotyrosine mAb, and anti-Shc mAb by repeated reprobing. *Arrowheads* indicate the positions of c-Src, tyrosine-phosphorylated p52 Shc, and p46/p52/p66 Shc, respectively. *C*, VSMC were pretreated with or without AG1478 (250 nM) for 30 min and stimulated with Ang II (100 nM) for indicated durations. After cell lysis, immunoprecipitation was performed with anti-EGF receptor pAb. Precipitates were analyzed by immunoblotting with anti-c-Src mAb (clone 28) and anti-EGF receptor pAb as indicated. *Arrowheads* indicate the position of c-Src and EGF receptor (*EGFR*), respectively. *DMSO*, dimethyl sulfoxide.

have also found that AG1478 (250 nM) inhibits Ang II-induced c-Fos expression and protein synthesis but not its intracellular Ca^{2+} mobilization or c-Jun induction in cultured rat VSMC.[2] These data further confirm the specificity of AG1478 and strongly support the general observations that AG1478 acts at the point of the EGF receptor transactivation induced by GPCRs, leading to specific inhibition of GPCR-coupled MAPK-dependent growth promoting signals, but does not interfere with functional coupling of GPCRs to other downstream kinases or signaling intermediates.

In addition, incomplete inhibition of Ang II-induced MAPK activation by AG1478 indicates that the activation is not exclusively mediated by the AG1478-sensitive pathway. The alternative activation signal(s) of MAPK by Ang II may involve other upstream transducers such as a novel Ca^{2+}-sensitive tyrosine kinase, PYK2

(30)/CAKβ (54)/RAFTK (55)/CADTK (56), Src family kinases (32–35) as discussed below, ErbB2 (31, 38), or protein kinase C (57). Further studies are required to determine relative contribution and possible cross-talks of these mechanisms leading to global growth promoting signaling.

Linseman *et al.* (25) showed that Ang II induced PDGF β receptor phosphorylation and subsequent complex formation with Shc, Grb2, as well as c-Src in cultured rat VSMC. However, the contribution of the PDGF β receptor to the MAPK activation by Ang II in VSMC is not likely. This view is supported by the observations that Ang II-induced MAPK activation was minimally affected by the selective PDGF receptor kinase inhibitor, AG1295, which almost completely abolished the PDGF-BB-induced MAPK activation (Fig. 1*C*) and that we could not detect the enhanced phosphotyrosine content of PDGF β receptor by Ang II in our VSMC (Fig. 5) during the time course in which the Ang II-induced maximum Ras (3~4 min) and MAPK activation (5 min) took place (36). However, Ang II-induced phosphorylation of the EGF receptor and its complex formation with the adaptors are detectable within 1 min. Given that the reported PDGF β receptor phosphorylation (25) was detected in 5 min, plateaued in 10 min, and sustained up to 120 min, it may signal to different downstream transducers rather than MAPK.

In cultured rat VSMC, Ang II has been shown to induce tyrosine phosphorylation of all three Shc isoforms of p46, p52, and p66 which subsequently form a complex with Grb2 (25). In the present study, tyrosine phosphorylation of Shc by Ang II was observed in the immunoprecipitates of the three Shc isoforms in which p52 phosphorylation was dominant (Fig. 6*B*). Furthermore, Ang II treatment resulted in association of these Shc isoforms to the GST-Grb2 fusion protein (Fig. 2*A*) and to the EGF receptor (Fig. 4*A*) in VSMC. In addition to Shc, Grb2 and Sos were also coprecipitated with the EGF receptor upon Ang II stimulation. Taken together with the recent findings that the expression of mutant Shc proteins defective in Grb2 binding displays a dominant negative effect on the pertussis toxin-insensitive MAPK activation induced by thrombin in fibroblasts (58), it is possible to speculate that the Ras and MAPK activation by Ang II may be at least partly mediated through Shc by linking the EGF receptor to a Grb2·Sos complex in VSMC. Some differences are noted in relative changes in the intensity of Shc bands immunoblotted by anti-phosphotyrosine mAb between Fig. 2*A* and Fig. 3 and between Fig. 2*A* blotted with anti-Shc pAb and Fig. 4*A* with anti-Shc mAb (the latter being needed to eliminate a thick rabbit IgG band). These may be due to a difference in efficiency of exogenous GST-Grb2 and endogenous Grb2 in binding Shc and also likely due to a difference in selectivity of anti-Shc pAb and mAb to each Shc isoform. It may also be possible that the relative difference in Shc band intensities is due to different affinity of each isoforms to Grb2 and the EGF receptor, respectively.

Ang II was reported to activate c-Src in cultured VSMC (43) which was proposed as an essential step for Ras activation by Ang II (44). Recently, Sadoshima and Izumo (34) reported that a Src family tyrosine kinase, Fyn, is activated by Ang II which recruits and phosphorylates Shc, leading to Ras activation in cardiac myocytes. We also found that Ang II increased transient association of the active c-Src with Shc which is contingent on Shc phosphorylation, suggesting a similar mechanism involving c-Src may operate the recruitment of Shc by Ang II in VSMC. Furthermore, the present study showed that Ang II and a Ca^{2+} ionophore enhanced the association of c-Src with the EGF receptor. Although the exact hierarchical order of activation of the kinases and adapters has yet to be clarified, given that c-Src has been shown to phosphorylate the EGF receptor

(51) and that the enhanced association of c-Src with the EGF receptor by Ang II was not affected by AG1478 (Fig. 6C), we submit the scenario in which the active c-Src phosphorylates the EGF receptor.

In the case of pertussis toxin-sensitive GPCR, the $\beta\gamma$ subunits of G protein play a crucial role in the Ras and MAPK activation which also involve Src kinases (35). Recently, it has been proposed that Src kinase is downstream of the wortmannin-sensitive phosphoinositide 3-kinase γ in this cascade (59). However, as we reported, Ang II induces pertussis toxin-insensitive Ras and MAPK activation in VSMC (36), and wortmannin has no effect on the Ang II-induced MAPK activation.[3] In agreement with our concept that c-Src phosphorylates the EGF receptor, a recent report by Luttrel *et al.* (60) showed that G_i-coupled receptor utilizes c-Src to phosphorylate the EGF receptor and for subsequent recruitment of the adaptors in MAPK activation. Thus, G_i- and G_q-coupled receptor-mediated MAPK cascades could converge on the Src kinase-operated EGF receptor transactivation.

In conclusion, we have demonstrated several lines of evidence that Ang II induces Ca^{2+}-dependent tyrosine phosphorylation of the EGF receptor which serves as docking sites for presumably pre-activated c-Src and downstream adaptors at the plasma membrane, leading to MAPK activation in cultured rat VSMC. The identification and characterization of the putative transducer(s) which directly sense intracellular Ca^{2+} mobilization to activate the kinases are under investigation.

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EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF

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Cross-communication between different signalling systems allows the integration of the great diversity of stimuli that a cell receives under varying physiological situations. The transactivation of epidermal growth factor receptor (EGFR)-dependent signalling pathways upon stimulation of G-protein-coupled receptors (GPCRs), which are critical for the mitogenic activity of ligands such as lysophosphatidic acid, endothelin, thrombin, bombesin and carbachol, provides evidence for such an interconnected communication network[1–4]. Here we show that EGFR transactivation upon GPCR stimulation involves proHB-EGF and a metalloproteinase activity that is rapidly induced upon GPCR–ligand interaction. We show that inhibition of proHB-EGF processing blocks GPCR-induced EGFR transactivation and downstream signals. The pathophysiological significance of this mechanism is demonstrated by inhibition of constitutive EGFR activity upon treatment of PC3 prostate carcinoma cells with the metalloproteinase inhibitor batimastat. Together, our results establish a new mechanistic concept for cross-communication among different signalling systems.

Epidermal growth factor receptor transactivation was identified as a critical element in GPCR-induced mitogenic signalling[1,5,6], and in regulation of various ion channels[7,3]. As the process is very rapid[1,7,9] and GPCR-induced release of EGFR ligands into the cell culture medium cannot be detected[5,8], EGFR transactivation has been proposed to be exclusively mediated through intracellular signals[3,4]. Even though platelet-derived growth factor receptors (PDGFRs) are not tyrosine phosphorylated upon treatment of Rat-1 cells with GPCR ligands[2], a chimaera EP-R (ref. 10) consisting
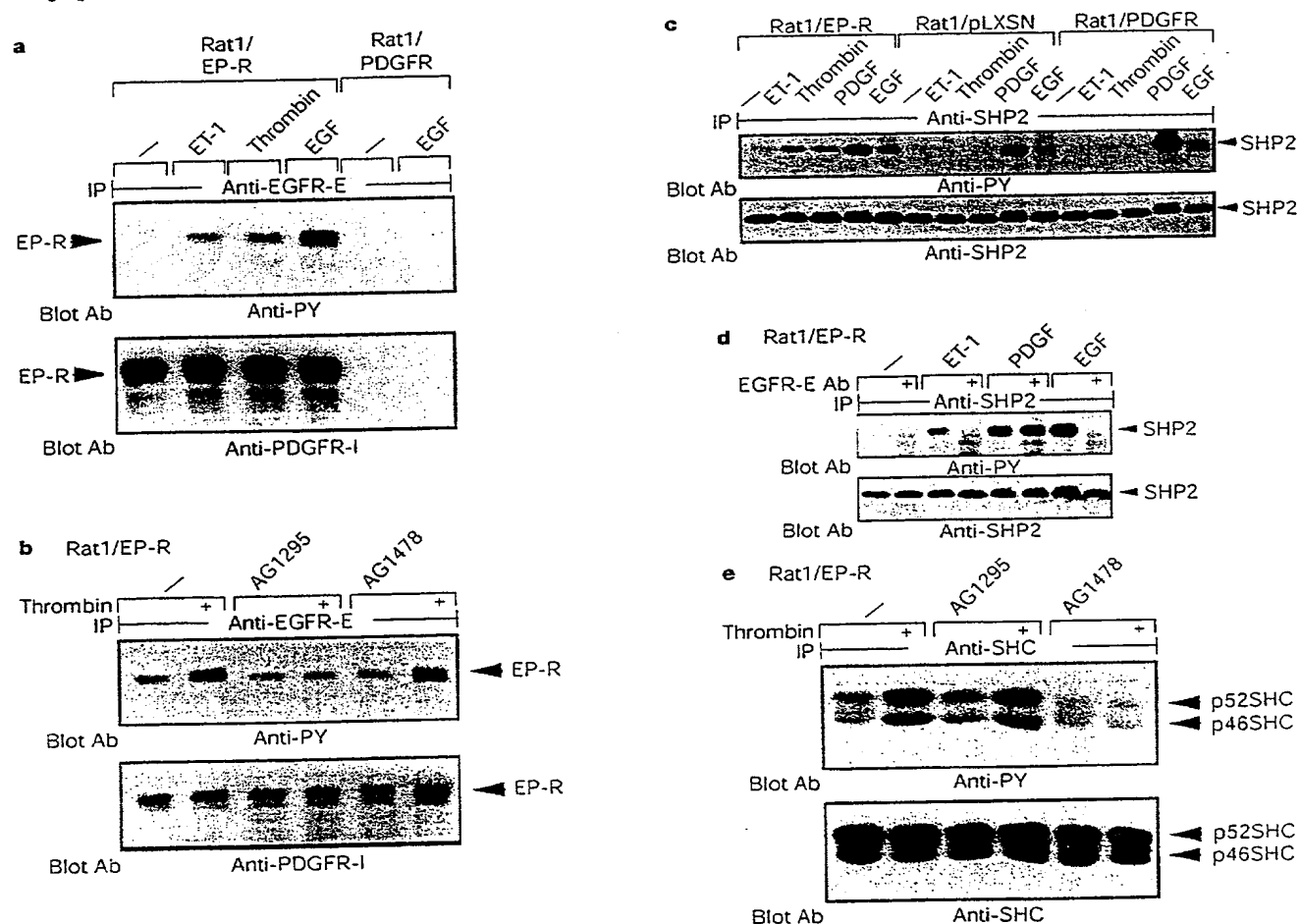
Figure 1 GPCR-induced EP-R transactivation redefines endogenous EGFR-mediated signalling to PDGFR-specific signals. Rat-1 cells stably expressing the EP-R chimaeric receptor (Rat-1/EP-R), the PDGFR (Rat-1/PDGFR) or vector alone (Rat-1/pLXSN) were treated with agonist for 3 min, and, after immunoprecipitation (IP), proteins were immunoblotted with anti-PY antibody (Ab) (4G10). Re-probing against the intracellular part of PDGFR (anti-PDGFR-I), SHP-2 or SHC, respectively, ensured precipitation of equal protein amounts. a, b, Rat-1/EP-R cells were stimulated with ET-1 (200 nM), thrombin (2 U ml⁻¹) and EGF (2 ng ml⁻¹) (a), or pre-incubated with tyrphostins as indicated (b) before thrombin stimulation, and EP-R was selectively precipitated with monoclonal antibody 108.1. c, d, Rat-1 cell lines were untreated (c) or pre-incubated for 1 h with EGFR-E Ab ICR-3R (20 μg ml⁻¹), stimulated with GPCR agonists, EGF or PDGF-BB (25 ng ml⁻¹) (d) and SHP-2 was precipitated. e, Rat1/EP-R were treated as in b and SHC was immunoprecipitated.

of an EGFR extracellular portion and the PDGFR transmembrane and cytoplasmic signalling domain is transactivated (Fig. 1a). Treatment of Rat-1/EP-R cells with the PDGFR inhibitor AG1295 (ref. 11), but not with the EGFR antagonist AG1478 (ref. 1), blocked thrombin-induced tyrosine phosphorylation of the chimaeric receptor (Fig. 1b). Figure 1c shows that EP-R transactivation results in a PDGF-characteristic downstream signal, as the SH2-domain-containing phosphatase 2 (SHP-2), a preferred mediator of PDGFR signalling[12], was tyrosine phosphorylated upon endothelin (ET-1) and thrombin stimulation of Rat-1/EP-R cells but not Rat-1 cells overexpressing the PDGFR. Pretreatment of Rat-1/EP-R cells with monoclonal antibody ICR-3R[13], which blocks ligand binding to the human EGFR, resulted in the complete inhibition of ET-1- and EGF-induced SHP-2 tyrosine phosphorylation, whereas the PDGF-mediated response was not affected (Fig. 1d), confirming that GPCR-induced transactivation of the EP-R chimaera depends on the extracellular EGFR domain. In contrast, tyrosine phosphorylation of SHC (Src homologous and collagen-like) proteins after thrombin stimulation was blocked by pretreatment of Rat-1/EP-R cells with AG1478, but remained unaffected by pre-incubation with the PDGFR antagonist AG1295 (Fig. 1e). This confirms that
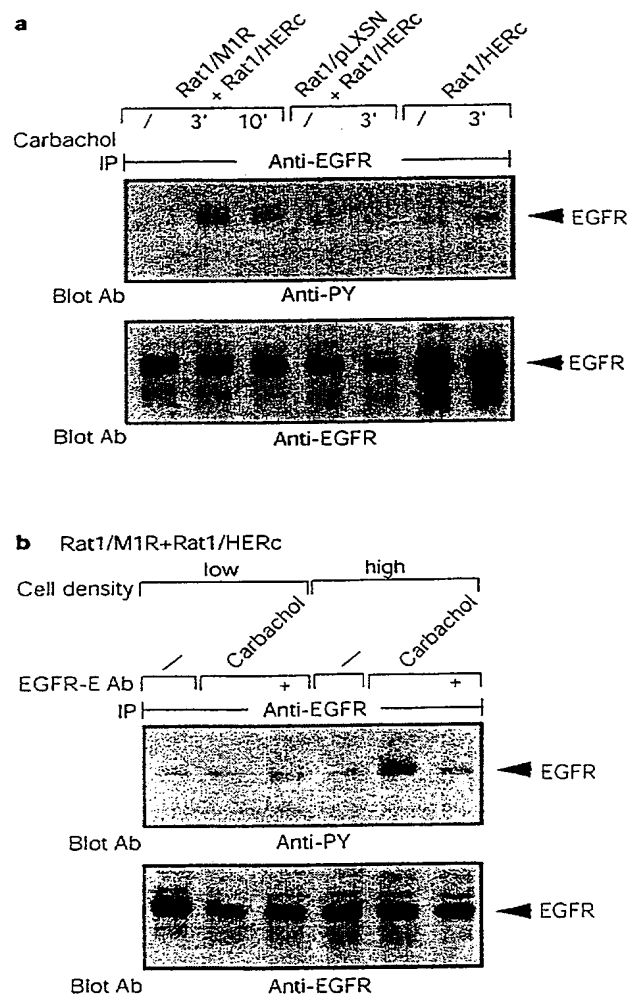
Figure 2 Carbachol-induced intercellular transactivation of the EGF receptor. Stable Rat-1 cell lines expressing either M1R or HERc and control cells were mixed in a 1:1 ratio. **a**, After stimulation with carbachol (1 mM), HERc was precipitated and immunoblotted with anti-PY antibody. **b**, Co-cultures of Rat-1/M1R and Rat-1/HERc cells were plated in different densities, pre-incubated with EGFR-E blocking antibody ICR-3R (20 μg ml⁻¹) and HERc was precipitated after 3-min stimulation with carbachol.
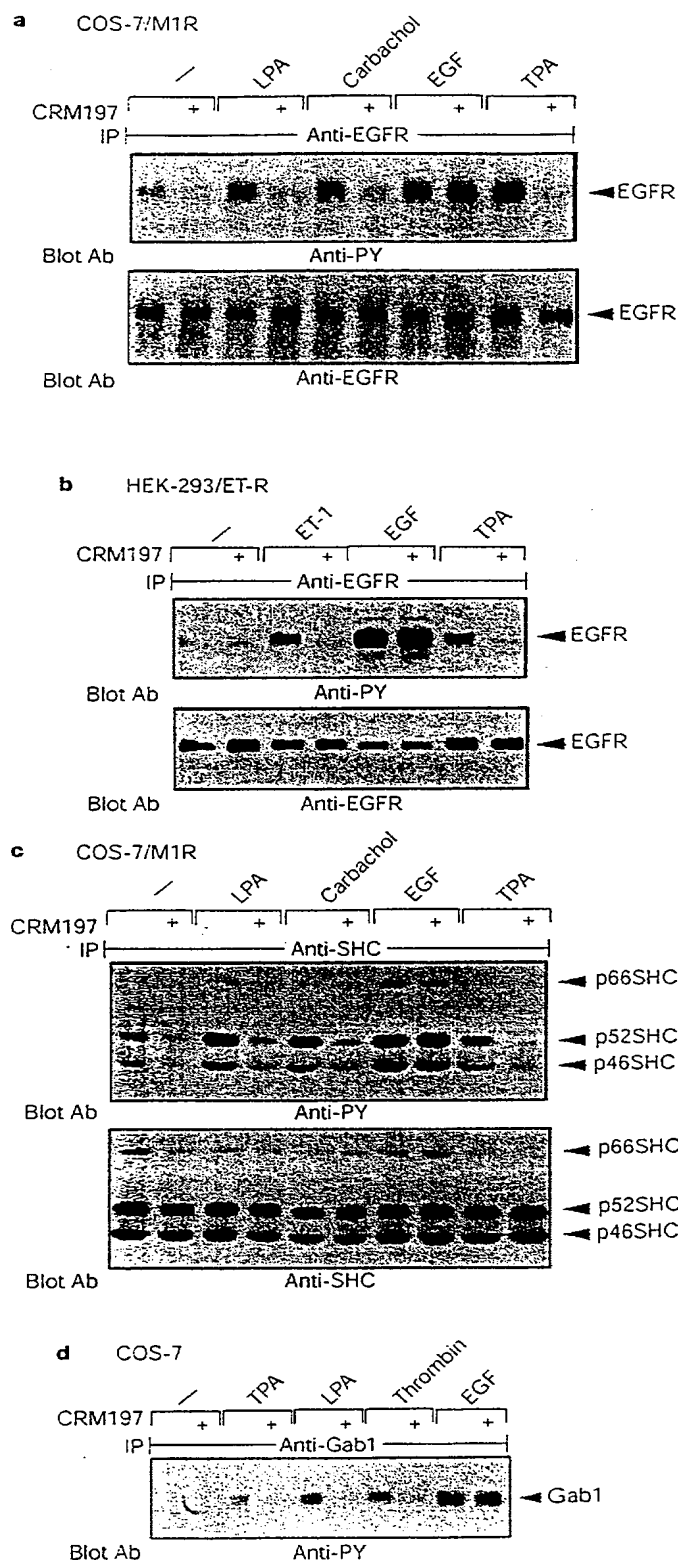
Figure 3 GPCR-induced EGFR transactivation and adapter protein tyrosine phosphorylation is dependent on HB-EGF function. COS-7 (**a, c, d**) and HEK 293 (**b**) cells, transfected with M1R and ET-R, respectively, and either CRM197 pre-incubated or untreated, were stimulated for 3 min with the GPCR agonists LPA (10 μM) or carbachol (1 mM), EGF (2 ng ml⁻¹) or 1 μM TPA (5 min) as indicated. Subsequently EGFR (**a, b**), SHC (**c**) or Gab1 (**d**) was immunoprecipitated and proteins were immunoblotted with anti-PY antibody (4G10).

letters to nature

thrombin transactivates endogenous rat EGFR in Rat-1/EP-R cells, which results in SHC tyrosine phosphorylation, whereas activation of the EP-R chimaera redefines thrombin stimulation to generate a PDGFR-characteristic SHP-2 signal.

To address whether the extracellular signal can act in a paracrine mode, we co-cultured Rat-1 cells overexpressing either the M1 muscarinic acetylcholine receptor (M1R) or the human EGFR (HERc). Stimulation of the Rat-1/M1R+Rat-1/HERc co-culture with the M1R agonist carbachol before immunoprecipitation with human EGFR-specific antibody 108.1 rapidly induced EGFR tyrosine phosphorylation (Fig. 2a). As neither of the control cells responded to carbachol, transactivation occurs between two cells. To investigate the influence of cell density, HERc was immunoprecipitated from subconfluent and confluent co-cultures after stimulation with carbachol. Figure 2b shows that EGFR tyrosine phosphorylation in response to an M1R agonist only occurred in confluent co-cultures and was inhibited by pre-incubation with the ICR-3R antibody. This further shows the requirement of the ligand-binding function of EGFR for intercellular signal transmission and the necessity of close cell–cell contact. Thus, we conclude that EGF-like ligands, synthesized as transmembrane precursors and converted to the mature form by proteolytic cleavage[14], may be involved in GPCR-mediated transactivation. The discrepancy between previous results, in which EGF-like ligands could not be detected upon GPCR activation[5,8], and our findings may be because upon proteolytic processing EGF-like ligands may remain associated with the heparan sulphate proteoglycan matrix before interaction with their high-affinity receptors.

Ectodomain shedding is induced by stimuli such as activators of heterotrimeric G-proteins, AlF$_4^-$ and GTPγS[15], as well as tetradecanoyl-phorbol-13-acetate (TPA) and the Ca^{2+}-ionophore ionomycin[16,17]. Ionomycin, which induces HB-EGF release in prostate epithelial cells[17], is a potent activator of EGFR transactivation in PC12 cells[18]. HB-EGF, a member of the EGF family, has the ability to bind to cell-surface heparan sulphate proteoglycans[19], which prevents the immediate release of the growth factor and increases the local growth factor concentration in the cellular microenvironment. Besides its function as a growth factor precursor, proHB-EGF serves as the high-affinity receptor for diphtheria toxin[20]. CRM197, a non-toxic mutant of diptheria toxin, inhibits strongly and specifically the mitogenic activity of HB-EGF[21]. We found that CRM197 pretreatment completely inhibits tyrosine phosphorylation of the EGFR induced by the GPCR agonists lysophosphatidic acid (LPA) and carbachol, as well as TPA in COS-7 cells (Fig. 3a). Inhibition was also observed for ET-1- or TPA-stimulated HEK 293 cells transiently transfected with the endothelin receptor (Fig. 3b). In contrast, EGF-induced receptor tyrosine phosphorylation was unaltered, demonstrating CRM197 specificity. Furthermore, complete abrogation of LPA- and carbachol-induced receptor tyrosine phosphorylation indicated that HB-EGF may be the only growth factor mediating EGFR transactivation in these cell lines.

To investigate the role of HB-EGF in the coupling of GPCR activation to Ras-dependent signalling pathways, we examined the effect of CRM197 on GPCR ligand and TPA-mediated SHC tyrosine phosphorylation[22]. Figure 3c shows that, in COS-7 cells, LPA-, carbachol- and TPA-induced SHC tyrosine phosphorylation was markedly reduced by CRM197 pretreatment, whereas the EGF-mediated response was not affected. Similarly, in COS-7 cells, tyrosine phosphorylation of the multidocking protein Gab1 in response to LPA or thrombin was not detected in the presence of CRM197 (Fig. 3d), which confirms its position downstream of the EGFR[2].

To examine whether proHB-EGF is proteolytically processed upon stimulation of GPCRs, we transfected plasmids containing VSV-tagged proHB-EGF in COS-7 cells together with the M1R or the bombesin receptor (BombR) and stimulated them with respective ligands for different times. TPA, a potent inducer of proHB-

Figure 4 GPCR-induced proteolytic processing of proHB-EGF and EGFR transactivation are critically dependent on metalloproteinase function. a, COS-7 cells were co-transfected with either M1R or BombR (0.5 μg each) and VSV-proHB-EGF (0.7 μg) and stimulated with carbachol (1 mM), bombesin (200 nM), TPA (1 μM) or EGF (2 ng ml^{-1}). ProHB-EGF was analysed with anti-HB-EGF antibody (upper panel); cleaved VSV-HB-EGF was monitored by anti VSV immunoblotting (lower panel). b, COS-7 cells transfected as in a were pre-incubated with batimastat (5 μM, 30 min), stimulated as indicated, and anti-VSV immunoprecipitates were subjected to anti-HB-EGF immunoblotting. c, Flow cytometric analyses of proHB-EGF in COS-7 cells treated for 10 min with LPA, TPA, EGF or batimastat pre-incubation after LPA stimulation. Control cells were labelled with FITC-conjugated secondary antibody alone. d, e, COS-7 cells, transfected with the M1R, untreated or BB-94 pre-incubated, were stimulated as in Fig. 3a, and EGFR (d) or SHC (e) was immunoprecipitated. Proteins were immunoblotted with anti-PY antibody (4G10). f, PC-3 cells were serum-starved for 36 h, pre-incubated with batimastat and stimulated for 3 min with bombesin, TPA or EGF (7 ng ml^{-1}) as indicated. EGFR was immunoprecipitated and immunoblotted with anti-PY antibody. g, Unstarved PC-3 cells were treated for indicated times with DMSO or batimastat, and EGFR tyrosine phosphorylation was monitored with anti-PY immunoblot.

EGF processing, and EGF were added as positive and negative controls, respectively. Figure 4a shows that, as previously described, proHB-EGF is expressed in form of heterogenous translation products of relative molecular mass 20–30K (ref. 16), which can be detected with antibodies against either the precursor or the VSV-tag. Stimulation with carbachol or bombesin led to a rapid breakdown of the growth factor precursor and proteolytic cleavage was concomitant with the appearance of the 9K VSV-tagged HB-EGF fragment containing the transmembrane anchor.

Because zinc-dependent metalloproteinases have been implicated in proHB-EGF shedding by TPA[23], we analysed carbachol-induced processing in the presence of batimastat (BB-94)[24], a potent inhibitor of metalloproteinases. As shown in Fig. 4b, BB-94 treatment reduced HB-EGF processing in response to carbachol, supporting our conclusion that metalloproteinases are critical elements in GPCR-induced HB-EGF generation.

To confirm that GPCRs induce proHB-EGF processing, we used an ectodomain-specific antibody and flow cytometry upon treatment of non-transfected COS-7 cells with LPA, TPA or EGF. Within 10 min after addition of LPA and TPA the content of cell-surface proHB-EGF was reduced, whereas EGF stimulation showed no effect (Fig. 4c). Consistent with the results in Fig. 4b, the modest LPA-induced effect was completely inhibited by batimastat.

Our results show that metalloproteinase-dependent cleavage of proHB-EGF is rapidly induced upon activation of GPCRs and consequently indicate a critical and general role of this process in EGFR transactivation. We therefore investigated the effect of the metalloproteinase inhibitor batimastat on GPCR- and TPA-induced EGFR transactivation. In COS-7 cells, BB-94 pretreatment completely abrogated LPA- and carbachol-induced tyrosine phosphorylation of the EGFR, as well as TPA-mediated receptor activation (Fig. 4d). As TPA- but not GPCR-mediated EGFR tyrosine phosphorylation is sensitive to protein kinase C inhibition in COS-7 cells (data not shown), it appears that at least two distinct metalloproteinase-dependent transactivation pathways exist. Finally, the general implication of proteolytic processing in EGFR transactivation and downstream signal transmission is shown by the complete abrogation of GPCR- and TPA-induced SHC tyrosine phosphorylation by batimastat (Fig. 4e).

Because of the well established role of EGFR family members in the pathogenesis of a variety of cancers and the physiological abundance of GPCR ligands such as LPA, we addressed the pathophysiological significance of transactivation with the human prostate cancer cell line PC-3, which has been reported to use EGFR-dependent pathways for growth promotion and which is also responsive to the GPCR ligand bombesin[25,26]. Figure 4f shows that, in PC-3 cells that were starved for 36 h, bombesin and TPA induce tyrosine phosphorylation of the EGFR which is completely

a COS-7/M1R or BombR+VSV-proHB-EGF

Carbachol Bombesin TPA EGF
/ 10' 30' / 10' 30' / 10' 30' 30'
IP Anti-VSV
—30K
—20K
Blot Ab Anti-HB-EGF
◄HB-EGF-1
Blot Ab Anti-VSV

b COS-7/M1R+VSV-proHB-EGF
TPA Carbachol
Batimastat / + +
IP Anti-VSV
—30K
—20K
Blot Ab Anti-HB-EGF
◄HB-EGF-I
Blot Ab Anti-HB-EGF

c
Relative cell number

200 160 120 80 40 0
Control
□ Unstimulated
■ LPA
10^0 10^1 10^2 10^3 10^4

200 160 120 80 40 0
Control
□ Unstimulated
■ TPA
10^0 10^1 10^2 10^3 10^4

200 160 120 80 40 0
Control
□ Unstimulated
■ EGF
10^0 10^1 10^2 10^3 10^4

200 160 120 80 40 0
Control
□ Unstimulated
■ Batimastat+LPA
10^0 10^1 10^2 10^3 10^4
Fluorescence intensity

d COS-7/M1R
LPA Carbachol EGF TPA
Batimastat / + + + + +
IP Anti-EGFR
◄EGFR Blot Ab Anti-PY
◄EGFR Blot Ab Anti-EGFR

e COS-7/M1R
LPA Carbachol EGF TPA
Batimastat / + + + + +
IP Anti-SHC
◄p66SHC ◄p52SHC ◄p46SHC
Blot Ab Anti-PY
◄p66SHC ◄p52SHC ◄p46SHC
Blot Ab Anti-SHC

f PC-3
Bombesin TPA EGF
Batimastat / + + + +
IP Anti-EGFR
◄EGFR Blot Ab Anti-PY
◄EGFR Blot Ab Anti-EGFR

g PC-3
DMSO Batimastat
6h 3h 1h 6h 3h 1h
IP Anti-EGFR
◄EGFR Blot Ab Anti-PY
◄EGFR Blot Ab Anti-EGFR

blocked by batimastat-treatment. Moreover, even the high constitutive phosphotyrosine content of the EGFR in unstarved PC-3 cells is reduced by long-term treatment with batimastat (Fig. 4g). Together, our results allow the conclusion that metalloproteinase-mediated precursor cleavage represents a direct link between BombR activation, constitutive tyrosine phosphorylation of the EGFR and proliferation of human prostate cancer cells.

Batimastat-sensitive metalloproteinases have been implicated in the processing of amphiregulin and TGFα precursors[27], and ADAM9, a member of the metalloproteinase-disintegrin family, has been reported to process proHB-EGF upon TPA treatment of Vero-H cells[23]. We were unable, however, to block EGFR transactivation with dominant−negative ADAM9 mutants in COS-7 and HEK 293 cells (data not shown), which leaves the identity of the proteolytic activity in this pathway unresolved.

Our findings identify the HB-EGF precursor and metalloproteinase activity to be critical pathway elements between GPCR signals and activation of the EGFR, and extend our understanding of the mechanisms that underlie the multiple biological processes known to be regulated by heterotrimeric G-proteins. The GPCR-induced EGFR transactivation mechanism represents a new paradigm because it entails three transmembrane signal transmission events. Ligand activation of heterotrimeric G-proteins by interaction with a GPCR results in an intracellular signal that induces the extracellular activity of a transmembrane metalloproteinase; this leads to the extracellular processing of a transmembrane growth factor precursor and release of the mature factor which, directly or through the proteoglycan matrix, interacts with the ectodomain of the EGFR and activates an intracellular signal. Our previous findings indicate that this pathway may be used by a variety of GPCRs in diverse cell types and that the preferred transactivation target is the EGFR and its relatives[1-4]. The pathophysiological relevance of this new mechanism in prostate cancer cells leads us to propose that EGFR transactivation through G-protein-mediated proteolytic growth factor precursor processing represents a general mechanism with broad significance. Moreover, as a great variety of bioactive polypeptides as diverse as tumour necrosis factor-α, FAS-ligand or L-selectin are processing products of transmembrane precursors[28] that have been connected to pathophysiological disorders, our fin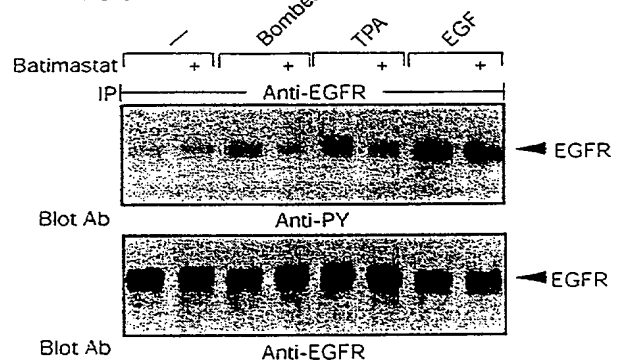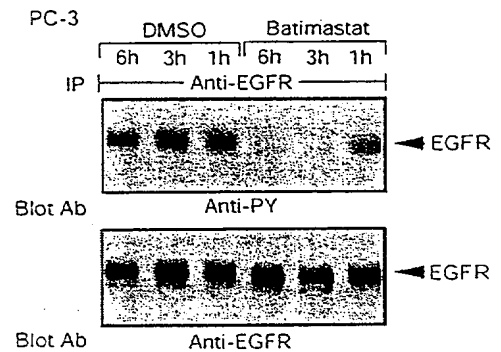dings shed new light on the importance of membrane-associated proteinases as targets for disease intervention strategies. □

Methods

Cloning and plasmids

Plasmids pcDNA1-BombR and pcDNA3-M1R have been described[1]. For stable expression of the M1R in Rat-1 cells the receptor was subcloned into pLXSN. proHB-EGF and the endothelin receptor were amplified by PCR from a MCF-7 or Rat-1 cDNA library and subcloned into pcDNA3-VSV or pcDNA3, respectively.

Cells and transfections

Rat-1 cells and COS-7 cells were grown and, respectively, infected and transfected, as described[1,2]. Rat-1HERc cells have been described elsewhere[1]. HEK 293 cells were grown in DMEM containing 10% FCS and transfections were carried out using the CaPO₄ method. CRM197 (10 μg ml⁻¹, Sigma) or batimastat (BB-94), (5 μM, British Biotech) were added 20 min before the respective growth factor. Tyrphostin AG1478 (250 nM, Calbiochem) and AG1295 (1 μM, Calbiochem) were added 15 min before stimulation.

Immunoprecipitation and western blotting

The antibodies against human EGFR (108.1), SHP-2, SHC and Gab1 have all been characterized[1,2,12,18]. Western blotting against the EP-R chimaera was carried out using a rabbit polyclonal α-hPDGFRβ antibody (Upstate Biotechnology). Cells were lysed and proteins immunoprecipitated as described[1]. To precipitate the VSV-tagged HB-EGF, a monoclonal VSV antibody (P5D4, Boehringer) in combination with Protein G-Sepharose was used; HB-EGF was detected with antibody C-18 (Santa Cruz). Because of the small size of pro-HB-EGF and the processed form of HB-EGF, we used the tricine SDS-PAGE system as described[29].

Flow cytometry analysis

COS-7 cells were seeded in 6-cm dishes for 20 h, before being washed and cultured for a further 24 h in serum-free medium until being treated with growth factors as indicated. After collection, cells were incubated with goat αHB-EGF antibody (R&D systems) for 30 min on ice. After washing with PBS, cells were incubated with FITC-conjugated rabbit anti-goat antibody (Sigma) for 20 min on ice. Cells were analysed with FACSCalibur (Becton Dickinson).

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CELLULAR FUNCTIONS REGULATED BY SRC FAMILY KINASES

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ABSTRACT

Src family protein tyrosine kinases are activated following engagement of many different classes of cellular receptors and participate in signaling pathways that control a diverse spectrum of receptor-induced biological activities. While several of these kinases have evolved to play distinct roles in specific receptor pathways, there is considerable redundancy in the functions of these kinases, both with respect to the receptor pathways that activate these kinases and the downstream effectors that mediate their biological activities. This chapter reviews the evidence implicating Src family kinases in specific receptor pathways and describes the mechanisms leading to their activation, the targets that interact with these kinases, and the biological events that they regulate.

CONTENTS

INTRODUCTION

Tyrosine phosphorylation has been implicated in the regulation of a variety of biological responses including cell proliferation, migration, differentiation, and survival. The protein tyrosine kinases involved in mediating these responses, as well as the receptors that activate them, encompass a diverse spectrum of proteins. Current evidence indicates that several distinct families of tyrosine kinases function in each of these responses and that additional complexity results from extensive cross-talk between different receptor pathways. One family of cytoplasmic tyrosine kinases capable of communicating with a large number of different receptors is the Src protein tyrosine kinase family (Src PTKs) (Figure 1).

The prototype member of the Src family protein tyrosine kinases was first identified as the transforming protein (v-Src) of the oncogenic retrovirus, Rous sarcoma virus (RSV) (Brugge & Erikson 1977, Purchio et al 1978). v-Src is a mutant variant of a cellular protein ubiquitously expressed and highly conserved through evolution (Stehelin et al 1976, Brown & Cooper 1996). A major breakthrough in understanding the function of the Src protein came from the finding that Src possesses protein tyrosine kinase activity (Collett & Erikson 1978, Levinson et al 1978). This evidence launched a search for related protein kinases, as well as investigations of the role of the viral and cellular forms of Src in the regulation of cell proliferation (for review, see Brown & Cooper
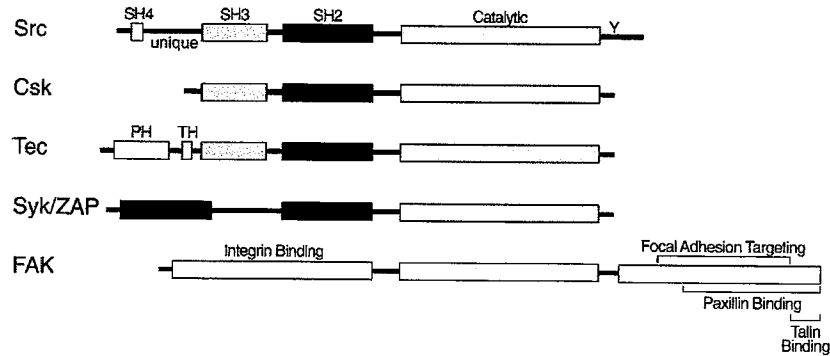
Figure 1   Domain structure of protein tyrosine kinases. Shown are the nonreceptor PTKs discussed in this chapter.

1996). Ten proteins were identified that contain structural features similar to Src and significant amino acid sequence homology: Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, Lyn, and the Frk subfamily proteins Frk/Rak and Iyk/Bsk (Cance et al 1994, Lec et al 1994, Thuveson et al 1995, Oberg-Welch & Welch 1995, Brown & Cooper 1996). Subsequent studies of these Src-related proteins led to the realization that these enzymes regulate many cellular events in addition to cell proliferation (e.g. cytoskeletal alterations, differentiation, survival, adhesion, and migration). This broad spectrum of activities is a consequence of the ability of these kinases to couple with many diverse classes of cellular receptors and many distinct cellular targets.

The focus of this review is on the structural and functional interactions between Src family kinases and cellular receptors and on receptor-induced biological activities regulated by these kinases. Before addressing these topics, we present a brief discussion of the expression of Src family kinases, their structural properties, and the role of individual domains in regulating the catalytic and binding activity of these kinases. For more detailed discussions of these topics, see a review by Brown & Cooper (1996).

Expression of Src Family Kinases

The Src PTKs can be subdivided into three groups based on their general pattern of expression (Table 1). Src, Fyn, and Yes are expressed in most tissues; however, individual kinases are expressed at elevated levels in certain cell types and some of these genes are expressed as alternatively spliced mRNAs in specific cell types. For example, Src is expressed ubiquitously; however, platelets, neurons, and osteoclasts express 5–200-fold higher levels of this protein (Brown & Cooper 1996).

Table 1 Expression of Src family kinases

Src	Ubiquitous; two neuron-specific isoforms
Fyn	Ubiquitous; T cell-specific isoform (FynT)
Yes	Ubiquitous
Yrk[a]	Ubiquitous
Lyn	Brain, B-cells, myeloid cells; two alternatively spliced forms
Hck	Myeloid cells (two different translational starts)
Fgr	Myeloid cells, B-cells
Blk	B-cells
Lck	T-cells, NK cells, brain

Frk subfamily
  Frk/Rak
  Iyk/Bsk
	Primarily epithelial cells

[a]Only found in chickens.

The second group of Src PTKs, Blk, Fgr, Hck, Lck, and Lyn, are found primarily in hematopoietic cells (Bolen & Brugge 1997). Both Lck and Lyn have also been detected in neurons, suggesting that these kinases may function in additional cell types. As in the case of Src and Fyn, alternate isoforms of some of these proteins have also been identified.

Frk-related kinases represent a subgroup of Src-PTKs (Frk/Rak and Iyk/Bsk). Frk and Iyk kinases share homology in all regions, including the unique region, and are expressed predominantly in epithelial-derived cells (Cance et al 1994, Lee et al 1994, Oberg-Welsh & Welsh 1995, Thuveson et al 1995).

These observations indicate that all cells are likely to express multiple Src PTKs and potentially multiple isoforms of an individual member. In addition, within a cell, these kinases can be found in many different subcellular locations. For example, Src has been found in caveolae, focal adhesions, and endosomes, whereas other members such as Fgr and Frk have been found in the nucleus (Kaplan et al 1992, 1994, Cance et al 1994, Robbins et al 1995, Thuveson et al 1995, Li et al 1996b, Lowell & Soriano 1996). Thus Src PTKs can function in many distinct cells and in distinct subcellular locations.

Structural Domains of Src Kinases

Src PTKs are 52–62 kDa proteins composed of six distinct functional regions (Figure 1): (*a*) the Src homology (SH) 4 domain, (*b*) the unique region, (*c*) the SH3 domain, (*d*) the SH2 domain, (*e*) the catalytic domain, and (*f*) a short negative regulatory tail (Brown & Cooper 1996). The SH4 domain is a 15-amino acid sequence that contains signals for lipid modification of Src PTKs (Resh 1993). The glycine at position 2 is important for addition of a myristic acid moiety, which is involved in targeting Src PTKs to cellular membranes. This

signal is absent in Frk (Lee et al 1994, Oberg-Welsh & Welsh 1995) but is present in Iyk. In addition, cysteine residues in the SH4 domain, which are present in all members except Src and Blk, are subject to palmitylation (Resh 1993). Frk and Iyk also have one of the conserved Cys residues, but whether these kinases are palmitylated has not been determined.

Following the SH4 domain is the unique domain which, as the name implies, is distinct for each member. The unique domain has been proposed to be important for mediating interactions with receptors or proteins that are specific for each family member. For example, sequences in the unique domain of Lck mediate its interaction with two T-cell surface molecules, CD4 and CD8 (Rudd et al 1988, Veillette et al 1988). Serine and threonine phosphorylation sites have also been identified in the unique domains of Src and Lck (Chackalaparampil & Shalloway 1988, Shenoy et al 1989, Morgan et al 1989, Winkler et al 1993). The precise function of these modifications is unclear but they may modulate protein:protein interactions or regulate catalytic activity.

The three domains that follow the unique region represent modular structures found in many classes of cellular proteins. The SH3 and SH2 domains are protein-binding domains present in lipid kinases, protein and lipid phosphatases, cytoskeletal proteins, adaptor molecules, transcription factors, and other proteins (Mayer & Baltimore 1993). The catalytic domain possesses tyrosine-specific protein kinase activity.

The SH3 domains of Src PTKs are composed of 50 amino acids (Pawson 1995, Cohen et al 1995). Alternatively spliced forms of Src, which contain 6- or 11-amino acid insertions in the SH3 domain, are expressed in CNS neurons (Brugge et al 1985, Martinez et al 1987, Sugrue et al 1990, Pyper & Bolen 1990). The SH3 domain is important for intra- as well as intermolecular interactions that regulate Src catalytic activity, Src localization, and recruitment of substrates.

SH3 domains bind short contiguous amino acid sequences rich in proline residues (Cohen et al 1995). All SH3 domain ligands contain a core consensus sequence of P-X-X-P; however, amino acids surrounding the prolines confer additional affinity and specificity for individual SH3 domains (Rickles et al 1995). SH3 ligands can bind in either a NH2 $\rightarrow$ COOH (Class I) or a COOH $\rightarrow$ NH2 (Class II) orientation (Yu et al 1994, Feng et al 1994). The SH3 binding pocket has two hydrophobic grooves that contact the core X-P-X-X-P sequence. A second region contacts the residues N-terminal (class I) or C-terminal (Class II) to the proline core. Binding affinities for SH3 domains and their ligands are in the micromolar range; however, such interactions may be strengthened in vivo by additional contacts between the target protein and other domains of Src (see below). Examples of proteins shown to interact with Src PTK SH3 domains either in vitro or in vivo include p68[sam], p85 phosphatidylinositol-3'

kinase (PI 3-K), and paxillin (Fukui & Hanafusa 1991, Liu et al 1993, Pleiman et al 1993, Prasad et al 1993a,b, Weng et al 1994, Taylor & Shalloway 1994).

A second modular domain that also controls the repertoire of proteins interacting with Src PTKs is the SH2 domain (Cohen et al 1995, Pawson 1995). Binding interactions mediated by the SH2 domain function in regulating the catalytic activity of Src PTKs, as well as the localization of Src or its binding proteins. In the case of Frk, a bipartite nuclear localization sequence is present in the SH2 domain and is likely to account for the ability of this kinase to localize to the nucleus (Cance et al 1994, Thuveson et al 1995). All SH2 domains bind to short contiguous amino acid sequences containing phosphotyrosine, and the specificity of individual SH2 domains lies in the 3–5 residues following the phosphotyrosine ($+1$, $+2$, $+3$, etc) (Songyang et al 1993, Pawson 1995). Amino acids preceding phosphotyrosine may also be important for regulating binding affinity (Bibbins et al 1993). Structural studies on Src family SH2 domains have shown that the ligand-binding surface of SH2 domains is composed of two pockets (Waksman et al 1993, Eck et al 1993). One pocket contacts the phosphotyrosine; the other pocket contacts the $+3$ amino acid residue following the phosphotyrosine. Src family kinases show a preference for leucine at this position (Songyang et al 1993). Examples of proteins shown to interact with the Src SH2 domain in vivo include the focal adhesion protein FAK (focal adhesion kinase), p130[cas], p85 PI 3-K, and p68[sam] (Fukui & Hanafusa 1991, Schaller et al 1994, Taylor & Shalloway 1994, Petch et al 1995).

Activation of Src Kinases

The SH2 and SH3 domains play a central role in regulating Src PTK catalytic activity. High-resolution crystal structures of human Src and Hck, in their repressed state, have provided a structural explanation for how intramolecular interactions of the SH3 and SH2 domains stabilize the inactive conformation of these kinases (see Figure 2) (Pawson 1997, Xu et al 1997, Sicheri et al 1997). The crystal structures include the SH3, SH2, and catalytic domains, and the negative regulatory tail. Both the SH3 and SH2 domains lie on the side of the kinase domain opposite the catalytic cleft. The SH3 and SH2 domains repress the kinase activity by interacting with amino acids within the catalytic domain, as well as with residues N-terminal and C-terminal, respectively, to the catalytic domain.

The SH3 domain interacts with sequences in the catalytic domain, as well as with sequences in the linker region that lies between the SH2 and catalytic domains (Sicheri et al 1997, Xu et al 1997). Although the linker region contains only a single proline residue, these sequences form a left-handed PPII helix and bind the SH3 domain in the same orientation as class II ligands. Two regions of the SH3 domain that flank the hydrophobic binding surface make contacts with the catalytic domain. Thus interactions with the linker region and the kinase

Basal Activity   Activated Kinase
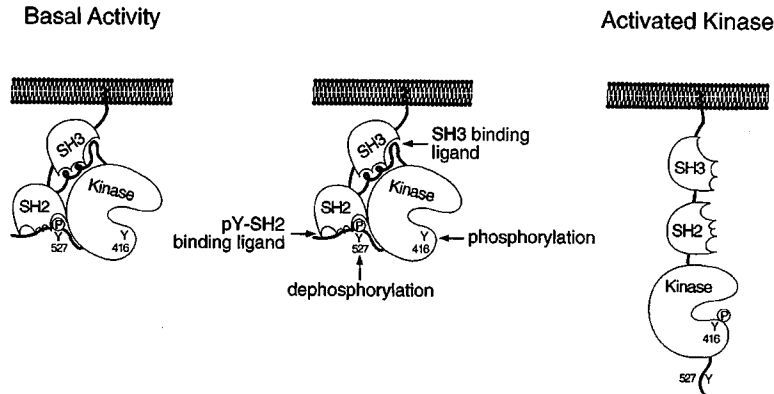
Figure 2   Mechanisms involved in activation of Src family kinases. The *left panel* shows a model of the structure of inactivated Src PTKs that are phosphorylated on the C-terminal tyrosine (Y527 in this model of Src). This model is based on the crystal structures of Src and Hck (Sicheri et al 1997, Xu et al 1997). The *middle panel* shows possible mechanisms involved in activation of Src PTKs. Y416 represents the autophosphorylation site in the activation loop of Src. The *right panel* represents a model for the activated state of Src in which the intramolecular interactions of the SH3 and SH2 domains are disrupted.

domain are likely to account for the SH3 domain's role in negatively regulating the catalytic activity of Src PTKs.

The SH2 domain interacts with pTyr 527 (Src) and adjacent residues in the negative regulatory tail (Brown & Cooper 1996). Y527 in c-Src, and the corresponding tyrosine in other Src PTKs, are the primary sites of tyrosine phosphorylation in vivo. This residue is phosphorylated by the cytoplasmic tyrosine kinase Csk (Cooper et al 1986, Okada & Nakagawa 1989, Nada et al 1991). Several lines of evidence indicate that loss of Y527 phosphorylation leads to activation of Src catalytic activity (Brown & Cooper 1996): (*a*) Mutation of Y527 results in constitutive activation of c-Src (Cartwright et al 1987, Kmiecik & Shalloway 1987, Piwnica-Worms et al 1987). (*b*) Y527 and several amino acids surrounding this residue are deleted in v-Src and similar truncations of c-Src cause activation of this enzyme (Reynolds et al 1987). (*c*) Disruption of the *csk* gene results in activation of at least three Src PTKs (Imamoto & Soriano 1993, Nada et al 1993). These results and others support a model whereby Csk-mediated tyrosine phosphorylation of the C-terminal tail promotes an intramolecular interaction between the SH2 domain and the phosphorylated tail, keeping the kinase in a closed, inactive conformation.

Some Src PTKs are not always phosphorylated at this negative regulatory tyrosine, yet remain relatively inactive. For example, in B cells, tyrosine

phosphorylation of the C-terminal tail of Lyn is barely detectable even though the catalytic activity of Lyn is not elevated. However, loss of Csk results in activation of Lyn (Nada et al 1993, Hata et al 1994). These results suggest that a balance between a tyrosine phosphatase and Csk is important for maintaining Lyn in an inactive state in unstimulated cells. In addition, other intramolecular interactions may be important for regulating the catalytic activity of Lyn. Although there are no real contacts between the SH2 and SH3 domain, the Src SH2 domain makes a few contacts with the large lobe of the catalytic domain, which may also contribute to repression of the kinase activity. Interactions between the SH3 domain and the kinase domain or upstream linker sequences may also contribute to repression of Lyn activity. For example, Hck which has been dephosphorylated, is further activated in the presence of an SH3 ligand (Moarefi et al 1997). Thus the role of C-terminal tail dephosphorylation in activation may vary in different systems.

Biochemical and structural studies of Src and other kinases suggest that the autophosphorylation site within the catalytic domain is also important for regulation of kinase activity. Analyses of the structures of the insulin receptor and protein kinase A (PKA) have shown that phosphorylation of analogous residues within the catalytic domain of these enzymes induces a conformational change that allows the kinase to assume an active conformation (Knighton et al 1991, Hubbard et al 1994, Johnson et al 1996). This site of phosphorylation corresponds to Y416 in c-Src, which is not phosphorylated in inactive wild type Src, but is constitutively phosphorylated in activated oncogenic Src mutants (Cooper et al 1986, Parsons & Weber 1989). Mutation of Y416 diminishes the transforming potential of both v-src and some oncogenic variants of c-Src, suggesting that phosphorylation of this residue may be important in vivo (Snyder et al 1983, Piwnica-Worms et al 1987, Kmiecik & Shalloway 1987, Kmiecik et al 1988). Structural studies of c-Src and Hck also suggest a possible regulatory role for phosphorylation of the catalytic domain (Pawson 1997, Xu et al 1997, Sicheri et al 1997). Protein kinase catalytic domains are composed of a small N-terminal lobe and a larger C-terminal lobe. The Src and Hck catalytic domains are in a closed conformation with the N- and C-lobes in close proximity, similar to that observed for active catalytic domains of PKA and the insulin receptor (Knighton et al 1991, Hubbard et al 1994, Johnson et al 1996, Pawson 1997). Although the Hck and Src catalytic domains appear to be in an active conformation, sequences in the N-terminal lobe are prevented from assuming a fully active conformation by constraints induced by the SH3/SH2/linker regions, the absence of tyrosine phosphorylation in the catalytic domain, and sequences in the C-terminal lobe. Thus phosphorylation of the activation loop tyrosine of Src PTKs is predicted to permit sequences in the N-terminal lobe to orient properly and allow the kinase to adopt an active conformation (under optimal conditions where restraints from SH3 and SH2 intramolecular interactions are disrupted).

Taken together, these studies suggest that there are multiple ways to activate Src family kinases (Figure 2). These include displacement of the intramolecular interactions of the SH2 or SH3 domains by high-affinity ligands or modification of certain residues, dephosphorylation of pY527 by a tyrosine phosphatase, or phosphorylation of Y416. As described below, more than one mechanism is often involved in Src activation in response to a single stimuli, and individual Src family members may be more sensitive to regulation by any one particular mechanism. This added complexity may influence temporal and spatial aspects of the regulation of Src PTKs and be important in determining which Src PTKs are activated by different stimuli. For example, in B cells, activation of a phosphatase that regulates the C-terminal tail of Lyn may not have a dramatic effect on Lyn activation (since only a small population of Lyn is phosphorylated at this site), but may be more important for activation of other Src PTKs expressed in these cells (e.g. Blk) (Hata et al 1994). In addition, regulation of the temporal aspects of Src kinase activation could affect the biological responses of the receptor. For example, transient or sustained activation of Src kinases could elicit distinct cellular responses following receptor activation. In PC12 cells, sustained MAP kinase activation correlates with a differentiation response, whereas transient MAP kinase activation correlates with a proliferative response (Marshall 1995).

In summary, the modular domains of Src PTKs endow these kinases with the ability to be regulated by and to communicate with a diverse group of proteins. The following sections provide an overview of the proteins that couple directly or indirectly with Src kinases. In particular, this review is focused on the different families of receptors that use Src PTKs to relay their messages and the downstream cellular events regulated by these kinases. Because of the vast amount of literature generated on the interactions between receptors and Src PTKs, we have concentrated on a subset of Src PTKs: Src, Fyn, and Lck. The review has been divided into three major sections that focus on (a) the interaction of Src PTKs with different receptor signaling pathways, (b) the cellular events in which Src PTKs are involved, and (c) the biological processes these kinases may regulate in vivo.

RECEPTOR PATHWAYS THAT COUPLE WITH SRC KINASES

Several strategies have been employed to determine if Src family kinases are involved in receptor-induced signal transduction pathways. These include investigations of whether receptor engagement leads to (a) coprecipitation or colocalization with the Src PTK, (b) activation of the Src PTK, or (c) phosphorylation of the Src PTK. In addition, investigations of whether inhibition of Src PTKs causes defects in receptor-induced changes in cell behavior have provided

evidence for involvement of these kinases in a receptor pathway. Because activation of one receptor pathway can lead to activation of other receptors, it has been difficult in certain systems to distinguish which receptor is responsible for Src kinase activation. For example, activation of G protein–coupled receptors (GPCRs), cadherins, integrins, and CAMs (immunoglobulin super family cell adhesion molecules), as well as stress pathways, result in activation or phosphorylation of receptor protein tyrosine kinases (RPTKs). Alternatively, activation of RPTKs or GPCRs can activate the binding activity of integrin receptors. In such systems, it is important to establish whether Src is activated following activation of the primary or secondary receptor pathway.

Although the receptors that couple with Src kinases are structurally and functionally distinct, a number of general conclusions can be drawn: (*a*) Src PTKs can interact directly or indirectly with receptors using a variety of different mechanisms. (*b*) Src PTKs can be activated by ligand engagement of the receptor, and activation can be mediated by multiple mechanisms. (*c*) Receptors not only regulate Src PTKs, but these kinases can also regulate the functional activity of receptors. The versatility of Src PTKs to function as both effectors and regulators of receptors allows these kinases to facilitate cross-talk between different receptors.

Immune Recognition Receptors and Major Histocompatibility Receptors

Immune recognition receptors (IRRs) play critical roles in immune responses to foreign substances and pathogens. This receptor family includes the T and B cell antigen receptors [TCR/CD3 and BCR) as well as Fc receptors (e.g. FcγRI, II, III, FcεRI, II) (Rudd et al 1993, Hulett & Hogarth 1994, Paolini et al 1994, Weiss & Littman 1994, Isakov et al 1994, Howe & Weiss 1995, Chan & Shaw 1995, DeFranco 1995, Bolen 1995, Roth & Wienands 1997, Daeron 1997). IRRs are composed of multiple subunits, some of which are involved in extracellular ligand interactions and others in coupling with intracellular cytoplasmic proteins. IRRs on T cells and B cells have served as prototypes for this family of receptors (Figure 3). Antigen recognition is mediated by surface IgM of the BCR or the α and β subunits of TCR/CD3. Intracellular signaling is mediated by the cytoplasmic domains of several receptor subunits (Igα and Igβ for the BCR and γ, δ, ϵ, ζ for TCR/CD3). Each of the latter subunits contains a shared sequence motif referred to as immunoreceptor tyrosine activation motif (or ITAM) defined by the sequence D- XX- Y-XX-L-X$_{7-8}$-Y-XX-L/I (Reth 1989). The tyrosine residues within this motif are phosphorylated following IRR engagement and play a critical role in recruitment of SH2 domain-containing kinases and other signaling proteins to the receptor complex.

TCR

BCR

CD4
(or CD8)

α β   ζ

γ δ ε

Lck   Fyn

Syk/Zap

Syk

Lyn
Blk
Fyn
Lck
Fgr
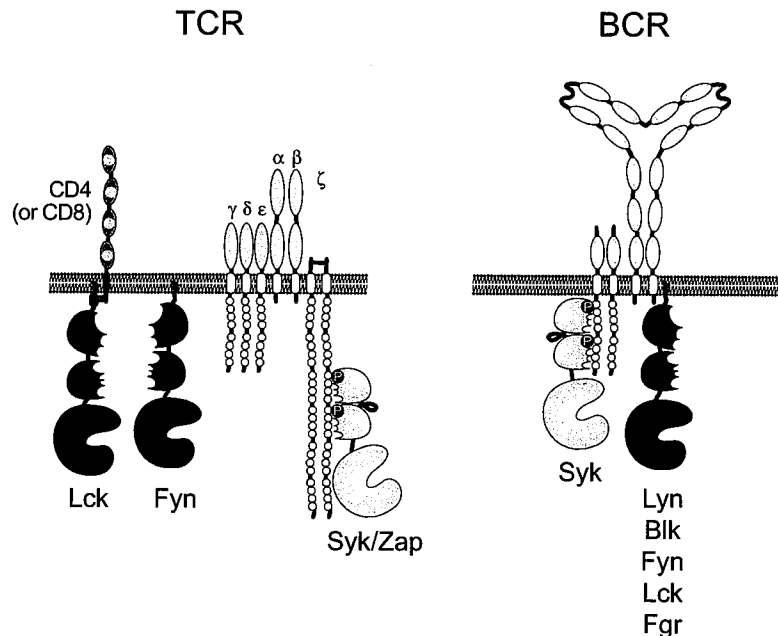
Figure 3   Components of the T cell receptor (TCR) and B cell receptor (BCR).

The specific kinases shown to couple with each member of the immune
response receptor family are indicated in Table 2. There is a great deal of
redundancy in Src kinase activation, both with respect to the ability of any
one family member to be activated by multiple receptors and the ability of one
receptor to activate multiple Src family kinases. This list of kinases activated
by each receptor is likely incomplete because the full spectrum of kinases for
each receptor has not been examined.

Antigen recognition by T cells involves coordination of the TCR/CD3 com-
plex with one of two coreceptors, CD4 or CD8, which bind to major histocom-
patibility (MHC) class I or II proteins. Because antigens are presented to T cells
in the form of peptides bound to the polymorphic cleft of MHC molecules, both
the αβ subunits of the TCR as well as CD4 or CD8 are required for recognition
of the antigen-MHC complex (Townsend & Bodmer 1989).

In this section, we focus on Src kinases involved in antigen recognition by
T cells because the role of protein tyrosine kinases is best characterized in this
system and the TCR/CD3:CD4/8 coreceptor complex provides a model for how
Src PTKs can coordinate signals transduced by two distinct receptors.

Table 2   Immune response receptor coupling with Src PTKs[a]

Receptor	Src PTK	Reference
TCR/CD3	Fyn, Lck	Rudd et al 1993, Isakov et al 1994, Weiss & Littman 1994, Bolen 1995, Howe & Weiss 1995
BCR	Lyn, Blk, Fyn, Fgr, Lck	Cambier & Jensen 1994, Desiderio 1994, Sefton & Taddie 1994, Penhallow et al 1995, Satterhwaite & Witte 1996
FcεRI	Lyn	Eisenman & Bolen 1990
FcεRII	Fyn	Sugie et al 1991, Maekawa et al 1992
FcγRIIA	Lyn	Bewarder et al 1996
FcγRIIb1	Fyn, Lyn	Sarmay et al 1995, Bewarder et al 1996
FcγRIIIA	Lck	Salcedo et al 1993
FcμR	Lck, Fyn, Lyn, Src	Rabinowich et al 1996

[a]Src family kinases are included in this table if there is any evidence suggesting that they can couple with an immune response receptor.

Src family kinases physically associate with both the TCR/CD3 and CD4/CD8 receptors. FynT coprecipitates with TCR/CD3 and its activity is stimulated following cross-linking of this receptor (Samelson et al 1990, 1992, Tsygankov et al 1992, Da Silva et al 1992). The FynT-TCR interaction is detected only if mild detergents are employed to maintain weak protein interactions. The precise nature of this interaction is not understood; however, the first 10 amino acids of FynT are necessary and sufficient to interact with the ζ TCR subunit, and transfer of this region from Fyn to Src conferred binding to ζ. FynT associates with other CD3 chains (ε, γ, ν) as well as ζ (Gauen et al 1992). Approximately 20% of FynT can be coprecipitated with TCR/CD3; however only 2–4% of total TCR/CD3 is associated with FynT (Sarosi et al 1992, Gassman et al 1992).

CD4 and CD8 directly couple with Lck, and cross-linking of these receptors leads to Lck activation (Rudd et al 1988, Veillette et al 1988, Barber et al 1989). This interaction, which is more stable in detergent extracts than the FynT-TCR interaction, is mediated by interactions involving C-X-C-P motifs from CD4 and CD8 and two Cys residues near the N terminus of Lck. The exact molecular nature of this interaction is unknown; however, it does not involve covalent bonding between the Cys residues (Shaw et al 1989, 1990, Turner et al 1990). Approximately 30–90% of Lck is stably associated with CD4 and CD8, depending on which population of T cells is examined and on the conditions employed for immunoprecipitation. An unrelated cytokine receptor, 4-1BB, which is induced following T-cell activation also possesses a C-X-C-P motif and has been shown to bind to Lck (Kwon et al 1987).

How are Src family kinases activated by IRRs? The mechanism of activation of Src kinases by immune response receptors is not completely understood

but involves a delicate balance between phosphorylation and dephosphorylation of these kinases. The protein tyrosine kinase Csk is important for negative regulation of Lck, Fyn, and other Src kinases that couple with IRRs. Loss of Csk leads to activation of Src family kinases (Imamoto & Soriano 1993, Nada et al 1993, Hata et al 1994) and prevents T and B cell maturation at an early stage of development (Gross et al 1995). Overexpression of Csk also suppresses TCR-induced protein tyrosine phosphorylation and IL-2 production in an antigen-specific mouse T cell line (Chow et al 1993) and prevents development of CD4+/CD8+ T cells when "knocked-in" to the *fyn* locus of *fyn-/-* mice (Takeuchi et al 1993, Kanazawa et al 1996). Expression of an activated Fyn mutant lacking the Csk phosphorylation site prevented Csk's inhibitory effects in a T cell line, suggesting that the Csk inhibition is mediated by suppression of Src kinases (Chow et al 1993).

The protein tyrosine phosphatase CD45 also plays a role in regulating Src family kinases through dephosphorylation. CD45 is able to dephosphorylate the C-terminal negative regulatory phosphorylation site (Mustelin et al 1990, 1992). CD45-deficiency in mice causes impaired development of CD4+/CD8+ T cells. T cells from these mice or cell lines lacking CD45 are defective in TCR stimulation of tyrosine phosphorylation, calcium mobilization, and IL-2 production, as well as anti-CD4 induction of tyrosine phosphorylation (Pingel & Thomas 1989, Koretzky et al 1990, 1991, Kishihara et al 1993). The defects in T cell signaling correlate with decreased catalytic activity of Lck and Fyn and increased phosphorylation of the C-terminal tyrosine (Ostergaard et al 1989, Volarevic et al 1990, Shiroo et al 1992, Mustelin et al 1992, Hurley et al 1993). CD45 deficiency can be overcome by co-ligation of CD4 and TCR, possibly from co-clustering of Lck and Fyn, which leads to kinase activation through transphosphorylation of the regulatory tyrosine in the catalytic cleft (Deans et al 1992). These results indicate that the status of Lck phosphorylation of the C-terminal tyrosine is balanced by the activity of Csk, CD45, and possibly other protein tyrosine phosphatases and that these regulatory enzymes play an important role in T cell receptor signaling.

Phosphorylation of the activation loop tyrosine is also important for activation of Src PTKs in T cells. Substitution of phenylalanine for this residue of Lck (Tyr 394) prevents activation by CD4 cross-linking (Veillette & Fournel 1990).

The pYXXL sequences within ITAMs resemble high affinity Src SH2-binding sites. Thus it is not surprising that Fyn and Lck have been reported to bind to these motifs in activated cells (Clark et al 1994, Flaswinkel & Reth 1994, Johnson et al 1995). It is possible that this interaction stimulates Src kinase activity by competitively interfering with the intramolecular negative regulatory interaction between the Src kinase SH2 domain and the phosphorylated C-terminal tyrosine residue. Because Src PTKs mediate phosphorylation of

ITAMs, this would not represent the primary mechanism for Src kinase activation through immune response receptors; however, it could contribute to further recruitment and activation of these kinases within receptor complexes.

Current models for the early events that are triggered following stimulation of the IRRs involve four different families of PTKs: Src kinases, Syk/ZAP, Tec kinases, and Pyk2. Src PTKs appear to be the primary kinases activated following engagement of these receptors and play a role in activation of the other PTK families (Isakov et al 1994, Weiss & Littman 1994, Bolen 1995, Chan & Shaw 1995, DeFranco 1995, Howe & Weiss 1995). Receptor clustering or dimerization leads to activation or recruitment of Src family kinases to the receptor complex. These kinases phosphorylate the tyrosine residues within the ITAM sequences of the receptor cytoplasmic domains. The phosphorylated ITAM sequences also serve as high-affinity binding sites for the tandem SH2 domains of Syk or ZAP, which redistribute to the receptor complex. Binding to ITAM sequences alone and/or phosphorylation by Src family kinases following binding leads to activation of the catalytic activity of Syk or ZAP. This activation leads to autophosphorylation of Syk/ZAP, which creates binding sites for interaction with SH2-containing proteins, and to phosphorylation of other cellular proteins, which transduce signals from the receptor. Activation of Tec kinases is also dependent on Src family kinases. Src kinase phosphorylation of Tec kinases leads to activation of autophosphorylation and exogenous substrate phosphorylation (Saouaf et al 1994, Rawlings et al 1996, Park et al 1996b). Lastly, activation of the FAK-related kinase Pyk2 (also called RAFTK) is also dependent on Fyn, as demonstrated by the absence of TCR-mediated Pyk2 phosphorylation or activation in Fyn-deficient mice (Qian et al 1997). Lck is not required for Pyk2 phosphorylation based on studies in Lck-deficient mice. Moreover, Fyn directly interacts with Pyk2 and activates its catalytic activity in T cells and in COS cells.

Because at least four PTK families are activated by IRRs, it is difficult to distinguish Src substrates from those of the other activated PTKs. However, there is significant experimental evidence indicating that Src kinases mediate phosphorylation of Syk/ZAP and Tec kinases, as well as IRR subunit ITAM sequences. The role of Src kinases in phosphorylation of ITAM residues has been shown by reconstituting the receptor complex in a transient expression system and through the use of somatic cell mutants of T cell lines. Phosphorylation of ITAM residues from a CD8-ζ fusion protein transfected into COS cells requires coexpression of a Src family kinase such as Fyn or Lck (Chan et al 1992). In contrast, ZAP expression in the absence of cotransfection of Src kinases was not sufficient to induce tyrosine phosphorylation of CD8-ζ. In addition, Jurkat T cell mutants lacking Lck are unable to phosphorylate ζ, and this activity is restored by transfection of Lck (Straus & Weiss 1992).

Two other proteins, LckBP1 and p68^{sam}, which are phosphorylated on tyrosine after TCR stimulation, also bind to Lck and can be coprecipitated with Lck from cell lysates (Takemoto et al 1995, Fusaki et al 1997). The identity of other Src substrates induced by IRRs is more ambiguous. PI 3-K has been shown to bind to Lck and FynT via both its SH3 and SH2 domains and may be a direct substrate of these kinases (Augustine et al 1991, Yamanashi et al 1992, Pleiman et al 1993). The adaptor protein Cbl and the inositol trisphosphate receptor (IP$_3$-R) are phosphorylated on tyrosine following TCR stimulation and coprecipitate with Fyn (Sawasdikosol et al 1996, Reedquist et al 1996, Tsygankov et al 1996, Tezuka et al 1996, Jayaraman et al 1996). Spleen cells from *fyn-/-* mice are deficient in TCR-induced Cbl phosphorylation, and Fyn overexpressing T cells show elevated Cbl phosphorylation, suggesting that Fyn is required for Cbl phosphorylation (Tezuka et al 1996). However, because Cbl also associates with ZAP and Syk, these PTKs may contribute to Cbl phosphorylation (Lupher et al 1996, Ota et al 1996, Panchamoorthy et al 1996). Other proteins phosphorylated on tyrosine in activated T cells include PLCγ, Vav, Shc, HS1, Lnk, ezrin, Slp 76, and p120^{rasGAP}, and its associated proteins p62 and p190. Other receptors such as CD5 and CD6 could also be substrates of any of the PTKs activated following T cell stimulation (Rudd et al 1993, Yamanashi et al 1993, Weiss & Littman 1994, Huang et al 1995).

Biochemical studies of T cells lacking Lck have allowed more precise dissection of the role of Lck in specific T cell signaling events (Straus & Weiss 1992, Karnitz et al 1992). TCR-induced phosphorylation of TCR-ζ, CD3-ϵ, and ZAP is defective in thymocytes from Lck-deficient mice (van Oers et al 1996a). In JCaM-1 Lck-deficient Jurkat T cells, which are defective in IL-2 production and calcium mobilization following TCR/CD3 stimulation, there is no detectable induction of tyrosine phosphorylation of ζ, ZAP, or other cellular proteins (Straus & Weiss 1992). Lck transfection can rescue the signaling defects in these cells. Although these cells express Fyn at levels close to the parental cells, this kinase does not appear to be sufficient for TCR signaling. Another line of Lck-deficient cells (derived from CTLL-2 cells) is profoundly deficient in cytolytic responses to TCR stimulation, but only modestly defective in induction of cell proliferation (Karnitz et al 1992). These results suggest that Lck is essential for activation of tyrosine phosphorylation through the TCR in certain populations of T cells. However, the specific mechanism for activation/recruitment of Lck to the TCR is not understood because Lck has not been shown to be associated with or activated by TCR clustering (Veillette et al 1989). It is possible that CD4/CD8-Lck may be recruited to the complex following TCR clustering; this has been reported in one T cell line (Burgess et al 1991). However, these JCaM1 cells express very low levels of CD4 and do not express CD8, so this Lck function may not involve CD4/CD8. It has been

suggested that Lck plays a role independent of CD4/CD8, most likely in phosphorylation of tyrosines in ITAM motifs, and that the availability of this distinct population of Lck for interaction with the TCR can affect the responsiveness of the receptor (Weiss & Littman 1994).

What is the physiological importance of Src kinases linking with both the TCR and CD4/CD8 proteins? Although cross-linking of the TCR/CD3 complex can lead to activation of IL-2 transcription, calcium mobilization, and DNA synthesis, co-clustering of CD4 and TCR/CD3 causes a dramatic increase in these responses (Eichmann et al 1987, Anderson et al 1987), and activation of T cell hybridoma cells by antigen-presentation leads to a 50–100-fold enhancement in responses relative to TCR cross-linking alone (Harding & Unanue 1990, Glaichenhaus et al 1991). The coordinate activation of Lck and Fyn through their interactions with CD4/CD8 and the TCR likely contributes to this synergistic activation of T cells. Under conditions of exposure to natural antigens in vivo, where only a small number of receptors are engaged (in some cases with low-affinity ligands), this may be critical for T cell biological responses.

There are several explanations for the enhanced responses induced by co-engagement of TCR/CD3 and CD4/CD8. One model derives from recent evidence that ZAP is constitutively associated with CD3-ζ in thymocytes (Watts et al 1994, Chan et al 1995, Wange et al 1995). Because ZAP activation (in contrast to Syk) is dependent on phosphorylation by Src family kinases, antigen-MHC stimulation of CD4/CD8-Lck may be required to trigger T cell responses by phosphorylating and activating ZAP. In addition, since only a small percentage of TCR/CD3 receptors are associated with Fyn (based on coprecipitation studies), CD4/CD8 co-clustering with TCR may play an important role in recruitment of a Src-family kinase to the TCR/CD3 complex to mediate ITAM phosphorylation and activate ZAP/Syk (Rudd et al 1993, Weiss & Littman 1994).

Other receptors expressed in T cells have also been shown to couple with Src family kinases and can contribute to the initial activation of T cells by antigen-presenting cells (e.g. CD2, CD28, Thy-1, Ly-6, LFA-1, CD43). CD2 has been reported to copurify with Lck and Fyn (Marie-Cardine 1992, Eljaafari et al 1994, Vitte-Mony et al 1994), and Lck is activated by CD2 ligation (Lacal et al 1990). Thy-1 and Ly-6 are glycosylphosphatidylinositol (GPI)-linked receptors that co-immunoprecipitate with Fyn and Lck (Brown 1993). CD43 cross-linking leads to association of Fyn with this receptor through the Fyn SH3 domains (Pedraza-Alva et al 1996). Other receptors, such as CD5, contain tyrosine residues that are phosphorylated after TCR stimulation and likely participate in antigen-stimulated activation events by recruiting signaling proteins like PI 3-K to the membrane via SH2-phosphotyrosine-mediated interactions (Raab et al 1994).

Thus Fyn and Lck are activated by antigen-MHC binding to the TCR and CD4/CD8. Both kinases participate in the earliest detectable receptor-mediated signal transduction events leading to activation of multiple downstream protein tyrosine kinases and other signaling proteins that mediate T cell activation events. Whether Src kinases play a direct role in any downstream cellular processes (e.g. secretion, cytoskeletal rearrangements, transcriptional activation, stimulation of DNA synthesis) or whether their role is primarily limited to phosphorylation of ITAMs and activation of other kinases that transduce signals responsible for these events remains to be elucidated. The role of Lck and Fyn in T cell development in vivo is discussed below.

Integrins and Other Adhesion Receptors

Adhesion to extracellular matrices and to other cells is mediated by a diverse family of receptors, the best-characterized being integrins, cadherins, selectins, and CAMs (Gumbiner 1993, Rosales et al 1995). Src kinases have been implicated in adhesion events regulated by these receptors by the evidence described below.

INTEGRINS   Integrins are heterodimeric receptors that mediate cell-matrix and cell-cell interactions. There are at least 15 α subunits and eight β subunits that associate with each other to generate a diverse family of receptors with distinct ligand specificities (Hynes & Lander 1992). Following engagement by their adhesive ligands, integrins transduce signals within the cell that regulate cell adhesion and spreading, migration, proliferation, differentiation, and other changes in cell behavior. Src was first implicated in integrin-regulated events by the ability of oncogenic v-Src to phosphorylate $\beta 1$ integrin, as well as several other proteins that are associated with integrin-nucleated focal adhesion complexes, e.g. paxillin, vinculin, talin, tensin, p130^{cas} and FAK (Sefton et al 1981, Hirst et al 1986, Pasquale et al 1986, DeClue & Martin 1987, Glenney & Zokas 1989, Reynolds et al 1989, Kanner et al 1990, Kanner et al 1991, Davis et al 1991, Sakai et al 1994). Subsequently, most of these proteins have been shown to be phosphorylated on tyrosine following natural engagement of different integrin receptors in non-transformed cells (Petch et al 1995, Nojima et al 1995, Clark & Brugge 1995, Schwartz et al 1995, Rosales et al 1995, Polte & Hanks 1995, Vuori & Ruoslahti 1995, Burridge & Chrzanowska-Wodnicka 1996, Lafrenie & Yamada 1996, Harte et al 1996). These early studies showing that integrin engagement induces tyrosine phosphorylation of a similar set of focal adhesion proteins as v-Src suggested that cellular Src PTKs were involved in integrin-induced tyrosine phosphorylation. Subsequent studies have shown that integrin engagement can activate Src kinase activity, that Src can localize to focal adhesion sites, and that Src associates with several proteins found in

focal adhesion complexes. This section discusses the evidence for Src kinase activation by integrins, possible mechanisms responsible for this activation, factors that affect Src localization to focal adhesions, and the nature of the interactions between Src and other focal adhesion proteins.

Src is activated transiently by engagement of integrins following cell attachment to a fibronectin matrix (Kaplan et al 1995). The mechanism responsible for Src activation has not been elucidated; however, this activation is preceded by dephosphorylation of the negative regulatory phosphorylation site pY527. Thus the initial activation of Src could be mediated by a tyrosine phosphatase that is activated by integrins or redistributed to cellular compartments containing Src. In addition, Src activation could be mediated, or at least stabilized, by interactions of the Src SH3 or SH2 domains with high-affinity binding sites on focal adhesion proteins. As discussed below, several focal adhesion proteins possess such Src SH2 and/or SH3 binding sites (including FAK, Cas, and paxillin), and several of these proteins coprecipitate with Src following integrin engagement.

The ability of Src to localize to focal adhesions is dependent on integrin-induced conformational changes that allow accessiblity of its SH2 and SH3 domains to other cellular proteins. When fibroblasts are plated on a fibronectin-coated surface, Src redistributes to newly formed focal adhesions following its activation (Kaplan et al 1995). Activation of Src and pY527 dephosphorylation is transient, peaking at 15 min; however, Src remains associated with the focal adhesions, suggesting that activation is not required for sustained localization at these sites. v-Src as well as activated mutants of c-Src or truncated, kinase-minus c-Src variants constitutively associate with focal adhesions (or podosomes, rosette-like adhesion sites found in transformed cells) when cells are plated in the absence of extracellular matrix (ECM) (Rohrschneider 1979, Shriver & Rohrschneider 1981, Krueger et al 1983, Kaplan et al 1994, Okamura & Resh 1994). These results suggest a model whereby integrin engagement leads to an unmasking of the Src SH3 and SH2 domains following pY527 dephosphorylation, which allows c-Src to associate with focal adhesion proteins (Figure 4). The SH2 and SH3 domains of mutant activated forms would not require ECM-induced unmasking since they would be constitutively open. Constitutive association of Src mutants with focal adhesions could lead to the morphological alterations in focal adhesions seen in v-Src-transformed cells and cells expressing the N-terminal truncation mutant of Src (1-251) (Kellie et al 1991, Kaplan et al 1994).

c-Src localization to focal adhesions requires myristylation and the SH3 domain, but not the SH2 or catalytic domains (Kaplan et al 1994). Although the SH3 domains and myristylation site are sufficient for Src focal-adhesion association (Kaplan et al 1994), the SH2 domain may participate in association with
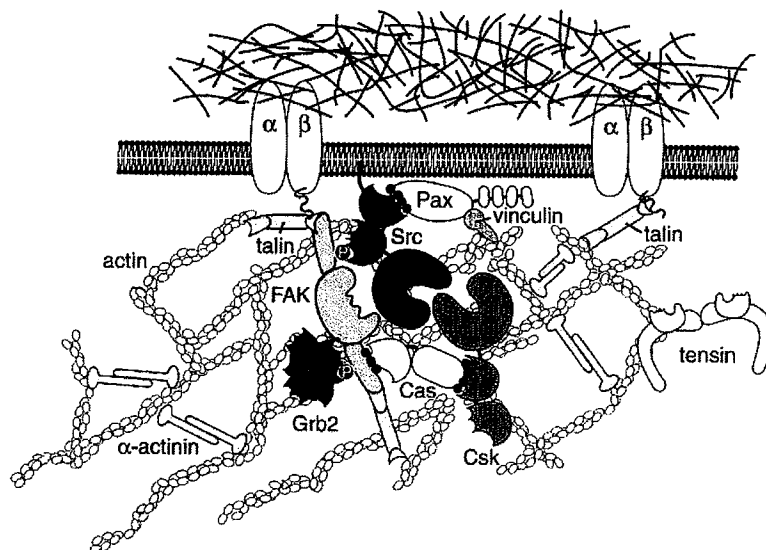
Figure 4   Src interaction with components of focal adhesion complexes. This figure was designed
to indicate that Src associates with focal adhesions following engagement of integrins by extracellu-
lar matrix. The intermolecular interactions displayed are hypothetical, based on known interactions
in vitro or co-immunoprecipitation from cell lysates and represent only a small number of proteins
within these complexes.

integrin complexes through interactions with tyrosine phosphorylated proteins
that localize to focal adhesions. One such protein is FAK (focal adhesion ki-
nase), a protein tyrosine kinase that is phosphorylated and activated following
engagement of many integrins (Schaller et al 1992, Guan & Shalloway 1992,
Kornberg et al 1992, Lipfert et al 1992, Schaller & Parsons 1993). FAK copre-
cipitates with Src and Fyn and this coprecipitation is dependent on an intact
FAK autophosphorylation site, Y397 (Schaller et al 1994, Cobb et al 1994,
Xing et al 1994, Eide et al 1995). Y397 is part of a pY-A-E-I motif that binds
to Src/Fyn/ Yes SH2 domains with a higher affinity than the pY527 site at the
C terminus of Src. Thus it is possible that the Y397 FAK autophosphorylation
site competes with the Src C-terminal phosphotyrosine site, thereby leading to
activation of Src (through disruption of the intramolecular interaction).

It has been postulated that Src association with FAK may facilitate Src-
mediated phosphorylation of other tyrosine residues on FAK, some of which
serve as binding sites for additional SH2-containing proteins (Schaller et al
1994) (Figure 5). For example, phosphorylation of Y397 is required for phos-
phorylation of FAK on Y925, a Grb2-binding site and phosphorylation of Y407,
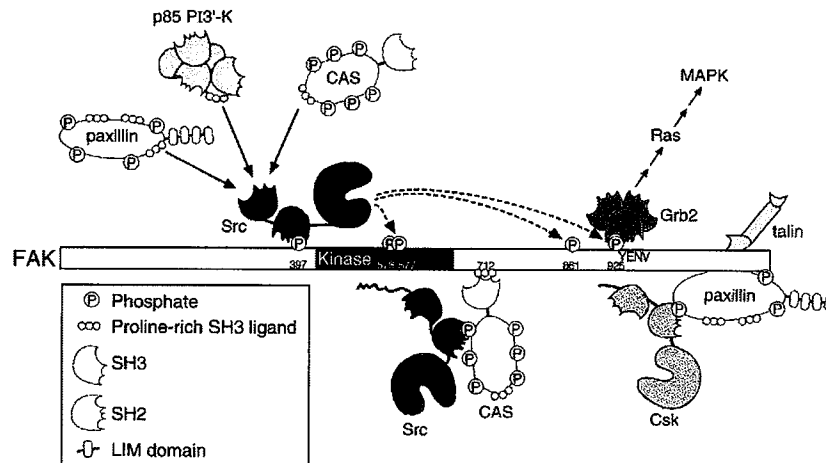
Figure 5   Interactions between FAK, Src, and other focal adhesion proteins.

Y861, Y576, and Y577, the latter two sites being required for maximal kinase activity of FAK and for FAK:p130^{cas} complex formation (Schlaepfer et al 1994, Calalb et al 1995, 1996, Schlaepfer & Hunter 1996, Polte & Hanks 1997). Thus Src or Fyn interaction with FAK may play an important role in FAK's interaction with other cellular proteins and in regulating FAK activity.

Several of the proteins that associate with FAK may be substrates of Src. FAK appears to serve as a scaffold to organize a network of signaling and cytoskeletal proteins. As shown in Figure 5, FAK associates with p130^{cas}, paxillin, Src, Grb2, and has also been shown to bind to p85:PI 3-K. p130^{cas}, paxillin and PI 3-K could also interact with Src through SH3 domain interactions (Hildebrand et al 1993, Polte & Hanks 1995, Chen et al 1995, Harte et al 1996, Burnham et al 1996, Vuori et al 1996, Schaller et al 1994). Tyrosine phosphorylation of paxillin following adhesion of cells to fibronectin requires Y397, suggesting a role for Src family kinases; however, because paxillin is phosphorylated in *src-/-* fibroblasts, it appears that Fyn or another PTK can compensate for the absence of Src (Schaller & Parsons 1995, Hamasaki et al 1996). Supporting this possiblity is the evidence that paxillin phosphorylation is significantly reduced in *src-/-; fyn-/-* double mutant cells (S Thomas, unpublished results). p130^{cas} is not phosphorylated in *src-/-* fibroblasts, suggesting that Src is involved in phosphorylation of this protein. However, the non-catalytic domain of Src may indirectly regulate p130^{cas} phosphorylation by mediating its localization to focal adhesions (Bockholt & Burridge 1995, Hamasaki et al 1996, Vuori et al 1996, Schlaepfer et al 1997; K Vuori, unpublished results). p130^{cas} phosphorylation can be

rescued by expression of a kinase-minus truncated form of Src (Schlaepfer et al 1997).

FAK may directly or indirectly recruit Csk to focal adhesions. Csk, which phosphorylates the negative regulatory tyrosine in the C-terminal tail of Src family kinases, also localizes to focal adhesions (Howell & Cooper 1994, Bergman et al 1995). Csk relocates to adhesion plaques when Src is activated, and redistribution to these sites requires the SH2 and SH3 domains of Csk (Howell & Cooper 1994). FAK and paxillin contain binding sites for the Csk SH2 and possibly SH3 domains (Sabe et al 1994). These data led to the proposal of a model suggesting that Csk is recruited to the focal adhesion by tyrosine phosphorylated focal adhesion proteins, possibly FAK or paxillin. Following this redistribution, Csk could phosphorylate Src on Y527, possibly leading to inhibition of Src catalytic activity (Sabe et al 1994, Howell & Cooper 1994).

A close relative of FAK, referred to as Pyk2, RAFTK, or CAKβ, undergoes an adhesion-dependent increase in tyrosine phosphorylation when cells are plated on fibronectin-coated dishes in some cell types, colocalizes with vinculin in an adherent human megakaryocytic cell line, and coprecipitates with paxillin in B cells (Lev et al 1995, Sasaki et al 1995, Li et al 1996a, Salgia et al 1996). The Y-A-E-I site is conserved between Pyk2 and FAK and has been shown to couple with Src in bradykinin and LPA signaling pathways in PC12 cells (Dikic et al 1996). Pyk2 is also regulated by Ca^{2+}, protein kinase C, and stress pathways (Lev et al 1995, Siciliano et al 1996, Tokiwa et al 1996, Yu et al 1996). Pyk2 expression is more restricted than FAK; however, in those cells in which both kinases are expressed, there may be redundant as well as unique functions of these kinases.

Other integrin receptors   Although integrin receptors for fibronectin are better characterized than others with respect to their coupling with Src PTKs, these kinases have been linked to other integrins as well. In platelets, Src associates with integrin-dependent detergent-insoluble cytoskeletal complexes following platelet aggregation, which is mediated by the integrin $\alpha_{IIb}\beta_3$ (Horvath et al 1992, Clark & Brugge 1993, Pumiglia & Feinstein 1993). This association with detergent-insoluble material is not detected in platelets from patients having mutations in the platelet integrin receptor $\alpha_{IIb}\beta_3$. Thrombin treatment of platelets causes an early integrin-independent activation of Src and pY527 dephosphoryation; however, Src does not associate with integrin complexes until after platelet aggregation mediated by $\alpha_{IIb}\beta_3$. Thus Src activation is uncoupled from integrin activation in this system.

Src is also activated by binding of osteoclasts and melanoma cells to osteopontin mediated by $\alpha_V\beta_3$ integrins. Src coprecipitation with this integrin following osteopontin binding requires the cytoplasmic tail of the α_V chain.

However, it is not known whether this interaction is direct or through other α_V-binding proteins (Hruska et al 1995, Chellaiah et al 1996). Src-deficient osteoclasts show defects in calcium signaling and in the induction of tyrosine phosphorylation following engagement of $\alpha_V\beta_3$, suggesting that Src-dependent events in integrin adhesion pathways may contribute to the resorption defect in Src-/- mice (R Baron, J Levy, unpublished results).

The role of Src family members other than Src and Fyn in integrin signaling has not been explored extensively. In neutrophils, engagement of β_2 integrins leads to activation of Fgr, which is enhanced by treatment with tumor necrosis factor (TNF) in a β_2-dependent fashion. Antibodies to β_2 block TNF activation of Fgr, and activation of Fgr in response to TNF does not occur in neutrophils from patients lacking β_2 (Berton et al 1994). In addition, analysis of neutrophils from hck-/-; fgr-/- mice showed that Hck and Fgr are required for signaling through leukocyte β_2 and β_3 integrins leading to neutrophil spreading and respiratory burst (Lowell et al 1996a). These results suggest that Fgr couples with β_2 integrins following their activation by TNF and binding to adhesive ligands.

Integrin coreceptors   Accumulating evidence suggests that other cellular membrane proteins couple with integrins to modulate their adhesive functions or merely to exploit the ability of integrins to communicate with the intracellular environment. Several GPI-linked receptors (uPAR, CD14 and FcγRIIIB/CD16), as well as TM4 proteins, GPIV, IAP, and caveolin, either couple directly with integrins or show functional interactions with them (Brown et al 1990, Huang et al 1991, Asch et al 1991, Fukasawa et al 1995, Petty & Todd 1996, Wary et al 1996, Li et al 1996b, Dorahy et al 1996, Gao et al 1996, Berditchevski et al 1996). Although the precise roles of these associated membrane proteins have not been defined, the co-modulatory activity of some may involve Src family protein tyrosine kinases. Several types of interactions have been reported: (*a*) physical interaction between the receptor and Src PTK [e.g. GPIV, which forms tight complexes with Fyn, Lyn and Yes in platelets (Huang et al 1991); caveolin, which interacts directly with c-Src and coprecipitates with α integrin subunits (Li et al 1996b, Wary et al 1996)]; and (*b*) indirect activation of Src kinases through integrins [e.g. IAP, which strongly enhances integrin-mediated tyrosine phosphorylation (Gao et al 1996)]. Integrin engagement has also been shown to lead to phosphorylation of receptor protein tyrosine kinases such as the platelet-derived growth factor (PDGF) receptors. Because Src kinases phosphorylate receptor protein tyrosine kinases (RPTKs) and act downstream from these receptors, Src could be involved in integrin-mediated coupling with these receptors (Wasilenko et al 1991, Hansen et al 1996, Peterson et al 1996).

CAMs   Neurite extension on L-CAM and N-CAM is reduced 50% in neurons from *src-/-* or *fyn-/-* mice, respectively (Beggs et al 1994, Ignelzi et al 1994), suggesting that these kinases may be involved in signaling through these CAM family receptors. Fyn coprecipitates with the NCAM140 isoform, which is predominantly localized in migrating growth cones, but not with NCAM180 or NCAM 120 (Beggs et al 1997). Src does not coprecipitate with any NCAM isoform. These results suggest that NCAM isoforms may couple with specific Src family kinases. In contrast, L1-CAM stimulation transiently activates both Src and Fyn in P19 embryonic carcinoma cells (Takayama et al 1997). Inhibition of these kinases through overexpression of Csk results in a defect in neurite fasciculation and cell-to-cell aggregation, implying that Src kinases may play roles in cell interactions mediated by CAM receptors. Src family kinases may be indirectly involved in CAM cell adhesion through their activation by FGF receptors. CAM-induced neurite outgrowth is dependent on activation of fibroblast growth factor (FGF) receptors (Williams et al 1994, Saffell et al 1997). As discussed below, Src associates with FGFR and is activated following treatment with FGF (Zhan et al 1994, Landgren et al 1995). In addition, microinjection of an antibody to Src inhibits FGF-induced neurite outgrowth in PC12 cells (Kremer et al 1991).

CADHERINS   Engagement of cadherin receptors has not been reported to activate Src-related kinases; however, Src has been shown to localize to cell-cell contacts with cadherin in aggregated cytotrophoblasts, and Src is activated significantly when cytotrophoblasts are maximally activated and starting to fuse (Rebut-Bonneton et al 1993). In addition, cadherins and their associated catenin proteins (α, β, plakoglobin) are phosphorylated on tyrosine in Src-transformed cells (Reynolds et al 1992, Matsuyoshi et al 1992, Hamaguchi et al 1993). v-Src expression causes a weakening of cadherin-mediated cell adhesion; however, it is difficult to definitively link this to catenin phosphorylation because many other cytoskeletal proteins are also phosphorylated in v-Src-expressing cells (Matsuyoshi et al 1992, Takeda et al 1995). These studies raise the question whether Src is involved in normal modulation of the cadherin adhesiveness or in downstream events that are triggered by cadherin receptor signaling. As with N-CAM and L-CAM, there is evidence suggesting that neurite outgrowth of rat cerebellar neurons induced by N-cadherin are also mediated by FGF receptors (Williams et al 1994). In other cell types, epidermal growth factor receptor (EGFR) colocalizes with cadherins at cell-cell adherens junctions, and in vitro association assays suggest that β-catenin mediates the interaction with EGFR through its highly conserved central core (Rebut-Bonneton et al 1993, Hoschuetzky et al 1994). Thus cadherins may recruit Src family kinases indirectly through activation of RPTKs.

SELECTINS   Selectin interactions with their glycoprotein ligands mediate initial steps in leukocyte adhesion to endothelial cells (Springer 1995). Selectin engagement of neutrophils and T cells induces tyrosine phosphorylation of several cellular proteins and activates MAP kinase and O_2^- synthesis (Waddell et al 1995, Brenner et al 1996). In T cells, selectin engagement results in activation of Lck, and induction of the cellular responses described above requires Lck since they were defective in Lck-deficient JCaM1.6 cells and rescued by transfection of Lck (Brenner et al 1996).

Src kinases are activated following engagement of multiple receptor pathways that regulate cell:cell and cell:matrix interactions. The roles of Src kinases in cellular events that are regulated by adhesion receptors are discussed below.

Receptor Protein Tyrosine Kinases

The receptor protein tyrosine kinase (RPTK) family is a diverse group of transmembrane proteins that bind to soluble and transmembrane ligands. Ligand binding results in stimulation of the catalytic activity of the receptor and initiates a cascade of signaling events that coordinate the diverse spectrum of biological responses mediated by these receptors (Kazlauskas 1994, van der Geer & Hunter 1994). RPTKs recruit multiple signaling proteins, including additional tyrosine kinases, to serve as downstream effectors. Among the kinases that participate in RPTK signaling are Src PTKs (Erpel & Courtneidge 1995).

Src family PTKs appear to communicate with many different RPTKs (Table 3). The biochemical connections between these different receptors and Src family kinases include phosphorylation of Src family PTKS, association with the RPTK, activation of Src PTKs, and phosphorylation of the RPTK.

Table 3   RPTKs coupled to Src PTKs

Receptor	Src PTK	Reference
PDGF-Rα/β	Src, Fyn, Yes	Kypta et al 1990, Twamley et al 1992; A Kazlauskas, personal communication;
EGF-R	Src, Fyn, Yes	Luttrell et al 1988, Sato et al 1995, Weernink & Rijksen 1995, Roche et al 1995b
FGF-R	Src	Zhan et al 1994
CSF-1R	Src, Fyn, Yes	Courtneidge et al 1993
NGF-R	Src	Kremer et al 1991
HGF-R	Src	Faletto et al 1993, Grano et al 1996
IR	Fyn	Sun et al 1996
IGF-R	Src	Kozma & Weber 1990, Peterson et al 1996
Neu (ErbB2)	Src	Muthuswamy & Muller 1995

PDGF RECEPTOR   The first RPTK to be linked with Src PTKs was PDGF-Rβ (Ralston & Bishop 1985). Treatment of fibroblasts with PDGF BB causes an increase in Src, Fyn and Yes catalytic activity. In addition, these kinases can bind directly to PDGF-Rβ and receptor activation induces both serine and tyrosine phosphorylation of Src and Fyn (Kypta et al 1990, Gould & Hunter 1988). Src PTKs are also activated in response to PDGF AA and can associate with the α-receptor (A Kazlauskas, personal communication).

Association   Src PTKs can bind directly to the PDGF-R (Kypta et al 1990, Twamley et al 1992). The Src SH2 domain binds to two phosphotyrosine residues in the juxtamembrane region of the PDGF-R (pY579 and pY581 for β and pY572 and pY574 for α) (Mori et al 1993; A Kazlauskas, personal communication). Mutation of both residues ablates the interaction between Src PTKs and the receptor. In the case of the β-receptor, interpretation of this result is complicated by the fact that this mutant receptor is catalytically compromised and, therefore, phosphorylation of other sites on the receptor is affected (Mori et al 1993). However, a similar mutation in the α-receptor ablates the interaction with Src PTKs without affecting its catalytic activity (A Kauzlaskas, personal communication). In addition, Src can directly bind a phosphopeptide comprising residues 572–589 of the β-receptor (Mori et al 1993, Alonso et al 1995). These results are consistent with a role for the juxtamembrane tyrosines of the receptor in mediating the interaction with Src PTKs.

Activation   Association of Src PTKs with the PDGF-R is likely to serve as an initial mechanism for activation of these kinases. Consistent with this hypothesis, Src can be activated in vitro with the tyrosine phosphorylated juxtamembrane peptide (β-receptor), and mutation of the juxtamembrane tyrosines in the α-receptor prevents PDGF-induced activation of Src (Mori et al 1993, Alonso et al 1995; A Kauzlaskas, personal communication). Thus PDGF-stimulated autophosphorylation would create a binding site for the Src SH2 domain, which would displace the intramolecular interaction with the negative regulatory tail and activate Src.

PDGF-induced phosphorylation of Src may also play a role in activation of Src PTKs. Src is phosphorylated on two serine residues, a tyrosine residue(s) in the N-terminal half and a tyrosine residue in the catalytic domain (Y416) in response to PDGF treatment (Ralston & Bishop 1985, Gould & Hunter 1988, Stover et al 1996, Broome & Hunter 1997).

One of the PDGF- and insulin-induced sites of serine phosphorylation is Ser12, which can be phosphorylated by PKC in vitro and is also phosphorylated in vivo in response to TPA treatment (Gould & Hunter 1988, Gould et al 1985). Phosphorylation of this residue does not appear to have any effect on the in vitro kinase activity of Src, and studies in other systems have shown that mutation

of Ser12 does not affect the transforming potential of an activated Src variant (Gould et al 1985, Parsons & Weber 1989). These studies suggest that Ser12 plays only a minor, if any, role in PDGF-mediated activation of Src. Because the other site of serine phosphorylation has not been mapped, the role of serine phosphorylation in PDGF-R-mediated Src activation remains elusive.

Two different tyrosine residues in the N-terminal half of Src have been identified as PDGF-induced phosphorylation sites. This discrepancy could be due to differences in the experimental approaches used to determine the site of phosphorylation. In one study Tyr213 was defined as the major site of tyrosine phosphorylation in PDGF-treated cells (Stover et al 1996). A tryptic peptide containing Tyr213 of Src can be phosphorylated by the PDGF-R in vitro, and this peptide co-migrates with a tryptic peptide obtained from Src isolated from PDGF-treated cells. Tyr213 is located within the SH2 domain of Src near the phosphopeptide-binding site. Src phosphorylated at this site in vitro by the PDGF-R has a reduced ability to bind a peptide corresponding to the negative regulatory C-terminal tyrosine (pYQPGE) but is unaffected in its ability to bind a phosphopeptide corresponding to a previously identified EGF-R binding site (pYDGIP). It has been postulated that the presence of the proline in the +2 position of the negative regulatory C-terminal peptide would promote an interaction between the glutamic acid in position +4 with Arg205 of the SH2 domain. Arg205 is in close proximity to Tyr213. Thus phosphorylation of Tyr213 could disrupt the SH2:pYQPGE interaction involving Arg205 and the glutamic acid in the COOH terminus of Src, resulting in a conformational transition from the closed to open state. Such a model argues that phosphorylation of Tyr213 would play a role in PDGF-mediated activation of Src. Interestingly, the corresponding residue in Lck (Tyr192) is tyrosine phosphorylated after T-cell activation and is proposed to play a role in Lck activation (Couture et al 1994). Mutational studies to determine whether loss of this site affects PDGF-mediated tyrosine phosphorylation or activation of Src will be important for testing this model and for definitively showing whether this residue is phosphorylated in vivo.

In contrast to the above results, in another study, Tyr138 was shown as the major PDGF-induced tyrosine phosphorylation site on Src (Broome & Hunter 1996, 1997). This site is phosphorylated in vitro by the PDGF receptor and is also phosphorylated in vivo after PDGF treatment of fibroblasts. In addition, mutation of Tyr138 abolishes (a) PDGF-mediated tyrosine phosphorylation of Src in vivo, (b) coprecipitaton of Src with the PDGF-R in NP40 cell extracts, and (c) PDGF-induced DNA synthesis. This mutant, however, can still be activated by the PDGF-R. Thus phosphorylation of Tyr138 is unlikely to play a role in the activation of Src by the PDGF-R; instead binding studies suggest that this site plays a role in regulating ligand binding to the SH3 domain. Tyr138 is

located in the peptide binding groove of the SH3 domain and likely contacts SH3 ligands. Phosphorylation of this residue results in a significant decrease in the ability of the SH3 domain to bind to either class I or class II peptide ligands (Broome & Hunter 1996). These results suggest that phosphorylation of Tyr138 is unlikely to play a direct role in PDGF-mediated activation of Src, but could regulate substrate specificity or, alternatively, provide a binding site for an SH2 ligand.

Although the site(s) of tyrosine phosphorylation in the N-terminal half of Src and its role in activation has not been completely resolved, tyrosine phosphorylation in the catalytic domain is likely to contribute to the PDGF-mediated increase in Src catalytic activity. Phosphorylation of the autophosphorylation site in Src (Y416) can be detected after PDGF treatment using an antiserum that specifically recognizes this phosphorylation site (X Zhou, S Sarkar & J Brugge, unpublished results). Since mutational studies suggest that phosphorylation of Y416 plays a role in regulation of its catalytic activity, phosphorylation of this tyrosine could be important for PDGF-induced activation of Src PTKs. Expression of the Y416F mutant in Src-deficient cells and analysis of PDGF-induced Src activation may help to address this issue.

EGF-RECEPTOR   Src is also involved in EGF-R signaling. Overexpression of Src enhances many different EGF responses including DNA synthesis, protein tyrosine phosphorylation, and tumor formation in nude mice (Luttrell et al 1988, Wilson et al 1989, Wilson & Parsons 1990, Chang et al 1995, Maa et al 1995). EGF treatment induces a two- to threefold increase in Src catalytic activity and translocation of Src to a Triton-insoluble fraction, another hallmark of Src activation (Sato et al 1995a, Weernink & Rijksen 1995).

Association/activation   Although Src has been shown to associate with the EGF-R in some cell systems, the nature of this interaction and its role in the initial activation is unclear. Sequences in the catalytic domain, as well as the SH2 domain of Src, have been proposed to mediate interactions with the EGF-R. A peptide corresponding to residues 413–431 from the catalytic domain can partially dissociate a constitutive interaction between the EGF-R and Src. There is no evidence, however, that this region of Src interacts directly with the EGF-R (Sato et al 1995a). In addition, peptides encompassing several receptor autophosphorylation sites bind to the Src SH2 domain in vitro. Tyr891 has also been shown to interact with Src both in vitro and in vivo (Sierke et al 1993, Stover et al 1995). Src can also phosphorylate Tyr891 and several other sites in vitro (Sato et al 1995, Lombardo et al 1995). These results, together with previous observations that the EGF-R is phosphorylated on novel tyrosine residues when Src is overexpressed with the EGF-R (Maa et al 1995), suggest that Src can potentially phosphorylate and bind to the EGF-R.

As with PDGF-induced Src activation, the initial activation of Src by EGF has been postulated to be mediated by Src's interaction with the receptor. Binding of a phosphorylated peptide encompassing pY891 to Src increases its catalytic activity; however, since Y891 has been proposed to be phosphorylated by Src, a conundrum exists as to how Src is initially activated (Stover et al 1995, 1996). One potential explanation is that another EGF-R family member (e.g. Erb B2 or Neu), which heterodimerizes with EGF-R, could be responsible for Src activation. For example, Neu can heterodimerize with the EGF-R and become activated after EGF stimulation. Src can bind to activated Neu in vivo, and an interaction between the Src SH2 domain and Neu has been shown in vitro (Muthuswamy & Muller 1995). Thus one possibility is that EGF would induce heterodimerization and activation of Neu and the EGF-R. Src would bind to tyrosine-phosphorylated Neu resulting in activation of Src. Src could then phosphorylate the EGF-R and provide a binding site for additional Src molecules. Determining whether the presence of Neu correlates with Src activation and EGF-R phosphorylation may help to resolve these issues.

FGF RECEPTOR   The FGF receptor can also recruit Src PTKs as downstream effectors in some cell types. An increase in Src family kinase autophosphorylation has been observed after FGF treatment of mouse fibroblasts and lung epithelial cells, but no association between Src and the FGF-R has been reported in these cell types (Landgren et al 1995). However, in NIH 3T3 cells, Src PTKs can associate with the FGF-R in vivo, and in vitro binding experiments suggest that this interaction is mediated by the SH2 domain of Src and autophosphorylation sites on the receptor (Zhan et al 1994). In addition, FGF also induces tyrosine phosphorylation of the v-Src substrate, cortactin, and an association between Src and cortactin is also detected after FGF treatment of NIH 3T3 cells (Zhan et al 1993, Schaller et al 1993). Whether Src PTKs are directly responsible for FGF-induced cortactin phosphorylation is unclear, but these studies suggest that Src PTKs also are involved in FGF-R signaling.

INSULIN RECEPTOR   While Src PTKs appear to interact directly with RPTKs in the systems described above, a slightly different variation has been observed in insulin receptor signaling. The insulin receptor is composed of an α subunit and two β subunits (Lee & Pilch 1994). The α subunit binds ligand resulting in activation of the β subunits. Activation of the insulin receptor results in tyrosine phosphorylation of a third critical component, an IRS (insulin receptor substrate) protein. The IRS proteins are a family of molecules that have been linked to a variety of receptors including the insulin/IGF-1 receptors and interleukin receptors (White 1994). These proteins contain multiple potential SH2-binding sites and thus have been proposed to act as a link between these

different receptors and SH2-containing effector proteins. One of the proteins shown to interact with IRS-1 after insulin stimulation is the Src PTK, Fyn (Sun et al 1996). As in the cases of the PDGF-R and EGF-R, this interaction is mediated by the SH2 domain of Fyn; however, thus far, no difference in Fyn kinase activity has been observed after insulin treatment. Understanding the precise role of this association in activation of Fyn and the role of Fyn in insulin-receptor signaling are issues to be resolved.

OTHER MECHANISMS OF ACTIVATION   Association of Src family kinases with the RPTK or its binding protein represents one mechanism of activation; however, other signaling molecules recruited to the receptor complex could be involved in RPTK-mediated Src activation (Erpel & Courtneidge 1995). For example, the tyrosine phosphatase SHP-2 binds to the PDGF-R and could activate Src PTKs by dephosphorylating the C-terminal negative regulatory tyrosine (Feng & Pawson 1994). EGF induces an increase in H_2O_2 that activates Src PTKs through an unknown mechanism (Bae et al 1997) (see stress section below). Thus additional mechanisms are likely to function in RPTK-mediated Src activation.

MODIFICATION OF RPTKS BY SRC PTKS   As mentioned above for the EGF-R, Src PTKs can also phosphorylate RPTKs. Studies on v-Src-transformed cells suggest that the β subunit of the IGF-1 receptor is a substrate for Src PTKs (Kozma & Weber 1990). In addition, loss of this receptor abrogates the ability of v-Src to induce transformation (Peterson et al 1996). While these studies do not definitively establish a role for the cellular counterpart of v-Src in regulation of RPTKs, tyrosine phosphorylation of RPTKs has been observed in other receptor systems where Src PTKs are activated. For example, Src may phosphorylate the PDGF receptor at a unique site (Hansen et al 1996), and engagement of some G protein–coupled receptors (GPCRs) can activate Src PTKs and induce tyrosine phosphorylation of the EGF-R in a Src-dependent manner (Luttrell et al 1997). Although the role of these phosphorylation events in receptor activation requires further analysis, these studies suggest that a complex regulatory loop exists between Src PTKs and RPTKs (Figure 6).

REQUIREMENT FOR SRC PTKS IN RPTK SIGNALING   RPTKs recruit multiple Src PTKs as well as other signaling molecules. Because some of the pathways regulated by these other signaling molecules that interact with RPTKs are functionally redundant with Src PTKs, redundancy can exist at many levels. For example, Src is associated with PI 3-K in PDGF-treated cells; however, PI 3-K can also bind the receptor directly (Kaplan et al 1987, Otsu et al 1991, Escobedo et al 1991). Thus while Src family kinases may be activated by multiple RPTKs, they may not be essential for biological events regulated by the receptor. Studies
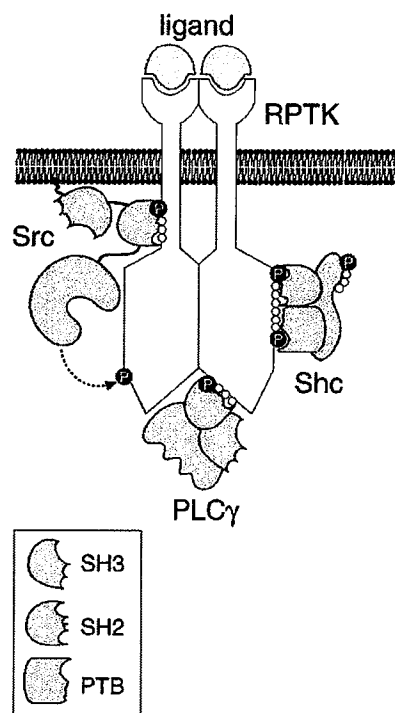
Figure 6   Interaction of RPTKs with Src and several representative SH2-containing signaling proteins. Src interacts with tyrosine phosphorylated motifs in RPTKs through its SH2 domain. Other proteins, such as Shc and PLCγ shown here, also bind to RPTKs through related SH2 domains. Src can also phosphorylate tyrosine residues on the receptor. The position of the Src SH2 binding motif at the juxtamembrane location represents the location of the Src binding sites on the PDGF receptor.

in some systems, however, suggest that Src family kinases do play a critical role in RPTK signaling pathways. For example, use of dominant-interfering mutants and antibodies against Src PTKs has demonstrated a requirement for these kinases in PDGF, CSF-1, EGF, NGF, and FGF signaling (Luttrell et al 1988, Wilson et al 1989, Kremer et al 1991, Twamley-Stein et al 1993, Roche et al 1995b). Although some of the results could be cell-type dependent, these studies indicate that Src PTKs are critical mediators in receptor tyrosine kinase signaling pathways (Figure 6).

G Protein–Coupled Receptors

G protein–coupled receptors (GPCR) form a large family of seven transmembrane-spanning proteins. These receptors are linked to heterotrimeric G protein

Table 4 G protein-coupled receptors linked to Src PTKs

Receptor	Src PTK	Reference
LPA	Src	Luttrell et al 1996
$\alpha 2_A A$	Src	Chen et al 1994, Luttrell et al 1996
Thrombin	Src, Fyn, Yes	Clark & Brugge 1993, Chen et al 1994, Daub et al 1996
M1	Src	Chen et al 1994
Angiotensin II	Src	Marrero et al 1995, Schieffer et al 1996
ET-1	Src	Simonson et al 1996
Bombesin	Src, Fyn, Yes	Rodriguez-Fernandez & Rozengurt 1996
Bradykinin	Src, Fyn, Yes	Rodriguez-Fernandez & Rozengurt 1996
Vasopressin	Src, Fyn, Yes	Rodriguez-Fernandez & Rozengurt 1996
FMLP	Lyn	Torres & Ye 1996
PAF	Src, Lyn, Fyn	Dhar & Shukla 1991, Kuruvilla et al 1994

complexes composed of α, β, and γ subunits (van Biesen et al 1996). Although initial studies suggested that these receptors engaged a set of signaling proteins distinct from those used by other receptor PTKs, increasing evidence suggests that there is great overlap between the effectors of GPCRs and RPTKs. For example, the MAP kinase cascade is activated after engagement of both RPTKs and GPCRs. Similarly, tyrosine phosphorylation also plays a role in certain GPCR signaling pathways. Src PTKs have been implicated in at least 11 different GPCR pathways that include G_i- and G_q-coupled receptors (Table 4). One $G_{\alpha o}$-coupled receptor may also be linked to Src PTKs (Diverse-Pierluissi et al 1997). Evidence for the involvement of Src PTKs in these pathways includes activation of the catalytic activity of one or more Src PTKs, association of these kinases with other signaling proteins, or a requirement for these kinases in certain GPCR-regulated events.

ACTIVATION   A two- to threefold activation of Src PTKs has been detected after engagement of multiple GPCRs. For example, endothelin-1 (ET-1) and platelet activating factor (PAF) stimulate the catalytic activity of Src, and thrombin increases the catalytic activity of Src, Fyn, and Yes (Dhar & Shukla 1991, Clark & Brugge 1993, Chen et al 1994, Daub et al 1996). Src shows an increased reactivity to an autophosphorylation-specific antibody after LPA treatment, suggesting that Src is activated in response to LPA (Luttrell et al 1996). Thus engagement of GPCRs can activate Src PTKs.

How GPCRs induce Src activation has not been elucidated. Although binding to the receptor could be a potential mechanism, thus far no direct association between the GPCR and Src has been observed. However, the heterotrimeric G protein subunits are important for Src PTK activation. Overexpression of $G_{\beta\gamma}$ subunits in COS7 cells induces a two- to threefold increase in autophosphorylated

Src, and activation of Src by G_i-coupled receptors is downstream of the $G_{\beta\gamma}$ subunits (Luttrell et al 1997). The activation of Src PTKs by $\alpha2_A$-adrenergic receptor ($\alpha2_A$AR) and thrombin is sensitive to pertussis toxin (PTX), which inactivates G_i (Chen et al 1994).

Protein tyrosine phosphatases   The above results implicate $G_{\beta\gamma}$ subunits in GPCR-mediated Src PTK activation. Because there is no evidence for a physical interaction between G protein subunits and Src PTKs, other proteins/molecules are needed to link these kinases to GPCRs. Studies on thrombin-, bombesin-, bradykinin-, and vasopressin-mediated activation of Src PTKs have suggested a role for tyrosine phosphatases. Thrombin treatment of platelets induces a transient, but rapid, dephosphorylation of Src, which precedes the increase in catalytic activity (Clark & Brugge 1993). In Swiss 3T3 cells, bombesin-, bradykinin-, and vasopressin-induced activation of Src is blocked by vanadate, a tyrosine phosphatase inhibitor (Rodriguez-Fernandez & Rozengurt 1996). These results suggest that a tyrosine phosphatase lies between the $G_{\beta\gamma}$ subunits and Src. At least two different PTPs have been implicated in signaling down-stream of G protein–coupled receptors. In platelets, both PTP1B and SHP1 are activated in response to thrombin treatment (Frangioni et al 1993, Li et al 1994, 1995). Although SHP1 has been proposed to have a negative regula-tory role in cytokine signaling, activation of SHP1 occurs very rapidly after thrombin stimulation (Li et al 1994, 1995, Imboden & Koretsky 1995). Thus in platelets, SHP1 could play a role in thrombin-induced Src PTK activation. A second tyrosine phosphatase, PTP1B, is activated by proteolytic cleavage (Frangioni et al 1993). This cleavage is dependent upon integrin engagement, which suggests that it is activated downstream of Src PTKs. PTP1B, therefore, is unlikely to be involved in the initial activation of Src PTKs but could partic-ipate in maintaining activation. In fibroblasts, a pertussis toxin-sensitive PTP activity has also been found to copurify with $G_{\alpha o/i}$ subunits, which suggests that PTPs can couple with heterotrimeric G proteins (Dent et al 1996). Identi-fication of this phosphatase activity and additional studies on the role of PTPs in GPCR-mediated signaling should help to resolve whether PTPs are involved in activating Src PTKs.

PI 3-K   Inhibitors of PI 3-K can block GPCR-mediated tyrosine phosphory-lation, suggesting that PI 3-K is important for activation of Src PTKs (Lopez-Illasaca et al 1997). An isoform of PI 3-K, PI 3-Kγ, can be activated by $G_{\beta\gamma}$ subunits. Because PI 3-Kγ contains a PH domain and because some PH do-mains can bind to $G_{\beta\gamma}$ subunits, $G_{\beta\gamma}$-mediated activation of PI 3-Kγ may result from binding of $G_{\beta\gamma}$ to PI 3-Kγ (Touhara et al 1994). Regardless of the mech-anism of activation of PI 3-K, production of phosphoinositol products of this kinase could be involved in subsequent activation of Src PTKs. One of the products of PI 3-K, PI 3,4,5P, can bind the Src SH2 domain (Rameh et al 1995).

Binding of PI 3,4,5P to the Src SH2 domain could displace interaction of the SH2 domain with the negative regulatory tail, leading to Src activation. Thus PI 3-K could play a role in the initial activation of Src PTKs in some GPCR systems. Determining whether PI 3,4,5P can directly activate Src PTKs will help strengthen this model.

FAK/Pyk2   Activation of FAK family kinases could also play a role in GPCR-mediated Src PTK activation. FAK is tyrosine phosphorylated following engagement of many different GPCRs (see below), and the FAK-related kinase Pyk2 also becomes tyrosine phosphorylated after LPA or bradykinin treatment of PC12 cells (Leeb-Lundberg & Song 1991, Sinnett-Smith et al 1993, Rankin et al 1994, Chrzanowska-Wodnicka & Burridge 1994, Polte et al 1994, Rozengurt 1995, Tippmer et al 1996, Dikic et al 1996). As described in the Integrin section, tyrosine phosphorylated Pyk2/FAK interacts with Src via the Src SH2 domain and a high-affinity binding site on Pyk2/FAK. In PC12 cells, LPA and bradykinin induce an association between Src and Pyk2, and Src associated with Pyk2 is activated. In addition, expression of Pyk2 in 293T cells results in Src activation, but this does not occur when a kinase-inactive or autophosphorylation mutant of Pyk2 is expressed (Dikic et al 1996). Thus Src binding to Pyk2/FAK could result in activation of Src. However, phosphorylation of Pyk2 is decreased when Csk is overexpressed, and bombesin can activate Src in the absence of FAK phosphorylation (Rodriguez-Fernandez & Rozengurt 1996, Dikic et al 1996). These results suggest that GPCR-mediated activation of Src occurs by both FAK family-dependent and -independent mechanisms.

RPTKs   As indicated above, tyrosine kinases other than Src PTKs appear to play a role in GPCR signaling. Activation of the LPA receptor, $\alpha 2_A$AR or thrombin receptor induces tyrosine phosphorylation of the EGF-R, and angiotensin II can induce tyrosine phosphorylation of the PDGF-R (Linseman et al 1995, Daub et al 1996). Phosphorylation of these receptors occurs through a nonautocrine mechanism, and dominant-interfering mutants of Src PTK or overexpression of a negative regulator of Src PTKs blocks tyrosine phosphorylation of the RPTKs (see below) (Luttrell et al 1997). Thus it is likely that Src PTKs are responsible for regulating tyrosine phosphorylation of the receptor tyrosine kinases. Phosphorylation of these receptors may provide docking sites for downstream signaling proteins involved in transducing the GPCR signal. Further studies are needed to elucidate the link between Src PTKs and RPTKs and understand the role of RPTKs in G protein–coupled receptor signal transduction.

REQUIREMENT FOR SRC PTKs   Although the precise role of RPTKs in GPCR-mediated signaling is unclear, a requirement for Src family kinases has been established for some G protein–coupled receptor pathways. Using inhibitory antibodies, dominant-interfering mutants or overexpression of Csk, Src PTKs

appear to be necessary in endothelin-1, LPA, and angiotensin II signaling (Simonson et al 1996, Schieffer et al 1996, Luttrell et al 1997). For example, antibodies to Src block angiotensin II-stimulated tyrosine phosphorylation. Interestingly, antibodies to Fyn or Yes have no effect, which suggests that Src plays a specific role in AT-II signaling (Schieffer et al 1996). In addition, LPA-induced tyrosine phosphorylation of Shc, EGF-R, and MAPK is inhibited by overexpression of Csk or expression of a dominant-interfering mutant of Src (Luttrell et al 1997). Similar studies on ET-1-mediated responses have shown a requirement for Src in *fos* induction (Simonson et al 1996). Thus Src PTKs are necessary for at least some GPCR-mediated events. Identification of downstream targets of these kinases and their role in the biological responses mediated by these receptors (e.g. proliferation or migration) should help to further define the function of Src PTKs in GPCR signal transduction.

Cytokine Receptors

The cytokine receptor superfamily, a large class of receptors, has been subdivided into two groups. This distinction is based on shared structural features of each class. Class I receptors contain an extracellular WSXWS motif (Bazan 1990). Examples include single-chain receptors such as the erythropoietin receptor (Epo-R) and prolactin receptor (Prl-R) and multisubunit receptors such as the interleukin 2 receptor (IL2-R) and granulocyte macrophage colony stimulating factor receptor (GM-CSF-R). The Class II receptor family, which is distinguished in part by its extracellular cysteine pairs, is composed of the antiviral factor receptors (interferon α, β, γ), the IL10-R, tumor necrosis factor receptor (TNFR), and the p75 NGF receptor. Both classes of receptors are capable of activating a signaling cascade that can regulate growth, differentiation, cell survival, and multiple specialized cell functions (Briscoe et al 1994). As in other receptor systems, tyrosine phosphorylation is induced following receptor engagement. The receptor subunits do not contain any intrinsic tyrosine kinase activity; however, at least three cytoplasmic PTK families have been shown to be involved in cytokine superfamily signaling: Jak kinases, SYK/ZAP 70 kinases, and Src PTKs (Ihle et al 1995). Src PTKs participate in signaling cascades initiated by many Class I receptors and at least one Class II receptor (Table 5). The evidence implicating Src PTKs in these receptor pathways includes cytokine-induced activation of Src PTKs, association of Src PTKs with the receptor subunits, cytokine-stimulated association of Src PTKS with other signaling components, and Src family kinase-mediated tyrosine phosphorylation of the receptor subunit(s) or downstream effectors.

IL-2 RECEPTOR   One of the first examples of Src PTK involvement in cytokine signaling came from analysis of IL-2 pathways (Briscoe et al 1994, Taniguchi

Table 5 Cytokine receptors coupled to Src PTKs

Receptor	Src PTK	Reference
IL-2	Fyn, Lck, Lyn	Hatakeyama et al 1991, Kobayashi et al 1993, Eljaafari et al 1995
IL-3	Fyn, Hck, Lyn	Kobayashi et al 1993, Anderson & Jorgensen 1995, Yousefi et al 1996
IL-4	Fyn, Lck	Ikizawa et al 1994, Wang et al 1996a
IL-5	Fyn, Lyn	Appleby et al 1995, Yousefi et al 1996
IL-6	Hck	Ernst et al 1994
IL-7	Fyn, Lck, Lyn	Seckinger & Fougereau 1994, Page et al 1995
IL-11	Src, Yes	Yang & Yin 1995, Fuhrer & Yang 1996a, Fuhrer & Yang 1996b
IL-12	Lck	Pignata et al 1995,
IL-15	Lck	Adunyah et al 1997
Prolactin	Fyn, Src	Clevenger & Medaglia 1994
G-CSF	Lyn	Corey et al 1994
GM-CSF	Lyn, Yes, Hck	Corey et al 1994, Linnekin et al 1994, Jucker & Feldman 1995, Yousefi et al 1996
TNF	Fgr	Berton et al 1994, Guy et al 1995
EPO	Src	Kitanaka et al 1994
Oncostatin M	Yes, Fyn, Src	Schieven et al 1992
4.1BB	Lck	Kim et al 1993

1995, Taniguchi et al 1995). IL-2 plays an important role in the proliferation of antigen-stimulated T cells but is also involved in other immune responses. In T cells, IL-2 stimulation results in the activation of the Src PTK, Lck (Horak et al 1991). Activation of Lck correlates with Ser/Thr phosphorylation of Lck, but the precise role of these modifications in Lck activation is unclear. Lck can also bind directly to the β subunit of the IL2-R (Hatakeyama et al 1991). Unlike interactions with other receptors, this interaction is mediated through the N-terminal part of Lck's kinase domain. This region of Lck interacts with an acidic region of the IL-2 receptor β subunit known as the A region. Receptors lacking this region are unable to bind or activate Lck, suggesting that this interaction is important for IL-2-mediated Lck activation. In addition, receptors that have mutations in a second region, the S region, also fail to activate Lck, although these receptors can still bind Lck. Thus the S region may bind a critical regulator of Lck. How Lck activation occurs upon IL-2 stimulation and whether the S region does, in fact, bind an activator of Lck remain to be determined.

Role of Src PTKs in IL-2 signaling   Whether Lck is required for IL-2 signaling is uncertain. The mutational studies described above indicate that the A region of the IL-2 receptor β_c subunit (which interacts with Lck) is not

required for IL-2-mediated proliferation. BAF-BO3 cells expressing an IL-2 receptor mutant lacking the A region can still proliferate in response to IL-2 (Miyazaki & Taniguchi 1996). In addition, natural killer (NK) cells derived from Lck-deficient mice can still proliferate in response to IL-2. In contrast to these results, certain T cell clones expressing IL-2R β-receptors with deletions in the A region have defects in their mitogenic response to IL-2 (Miyazaki & Taniguchi 1996). Thus Lck may be required for IL-2 signaling in specific T cell populations. The limited requirement for Lck function in IL-2 signaling could be due to the redundant functions of Src PTKs. Other Src PTKs (e.g. Fyn and Lyn) have been found to interact with the IL-2Rβ subunit in different cells, suggesting that multiple Src PTKs can function in IL-2 signaling pathways (Kobayashi et al 1993).

OTHER CYTOKINE RECEPTORS   While the requirement of Src PTKs in IL-2 signaling may be unresolved, Src PTKs have been shown to couple with several other cytokine receptors and to play a critical role in some of these cytokine receptor pathways. For example, studies on IL-5 signaling in B cells has shown that at least two kinases, Lyn and Fyn, are activated (Appleby et al 1995, Yousefi et al 1996). Both kinases can also associate with the β subunit, although the regions important for this association are unclear. Analysis of B cells from *fyn*-deficient mice indicate that loss of *fyn* results in defects in IL-5 signaling (Appleby et al 1995). In K562 cells, expression of anti-sense Src mRNA decreases EPO-induced proliferation and blocks hemoglobin synthesis and glycophorin expression (Kitanaka et al 1994). Where Src fits in the EPO-R signaling cascade, however, has not been determined. Regardless, these studies indicate that Src PTKs can function downstream of the cytokine receptor superfamily and together with other cytoplasmic PTKs form a tyrosine kinase network that mediates effects on growth, differentiation, and cell survival. Additional studies determining how Src PTKs are activated, the precise pathways they regulate, and how they are linked to other cytoplasmic PTKs should provide insight into the function of these kinases in cytokine signal transduction.

GPI-Linked Receptors

A structurally unique class of receptors that couples with Src family kinases are GPI-linked receptors. These receptors are extracellular proteins anchored to the outer leaflet of the plasma membrane via a GPI moiety (Englund 1993). GPI-linked receptors have been best characterized in hematopoietic cells; however, they are expressed in many cell types (Rudd et al 1993). Engagement of these receptors induces a broad spectrum of phenotypic alterations in cells, including migration, neurite extension, and proliferation (Rudd et al 1993, Bohuslav et al 1995, Zisch et al 1995). Several of the GPI-linked receptors have been shown

Table 6 GPI-linked receptors coupled to Src PTKs

Receptor	Src PTK	Reference
Thy-1	Fyn, Lck, Lyn	Stefanova et al 1991, Thomas & Samelson 1992, Narisawa-Saito et al 1996
Ly-6	Lck	Stefanova et al 1991
CD14	Lyn, Hck, Fgr, Lck	Stefanova et al 1991
CD48	Lck	Stefanova et al 1991
CD24	Lck	Stefanova et al 1991
CD55	Fyn, Lck	Shenoy-Scaria et al 1992
CD59	Lck	Stefanova et al 1991
F11	Fyn	Zisch et al 1995
F3	Fyn	Olive et al 1995
uPAR	Fyn, Lyn, Hck, Fgr	Bohuslav et al 1995

to cause an induction of tyrosine phosphorylation and activation of Src family PTKs (see Table 6). The mechanism whereby these receptors couple with PTKs and transduce signals to the intracellular environment is not understood. Because they lack transmembrane and intracellular domains, GPI-linked receptors are incapable of conventional signaling to the cytoplasm. The evidence connecting GPI-linked receptors to Src PTKs and the proposed models for how these proteins couple with each other are discussed below.

CD14 Lipopolysaccharide (LPS) binds to a LPS-binding protein (LBP) and the GPI-linked protein CD14 (Ulevitch & Tobias 1995). LPS treatment of macrophages results in an increase in tyrosine phosphorylation and activation of three Src PTKs: Lyn, Hck, and Fgr (Stefanova et al 1991). The role of Src PTKs in LPS signaling may be dispensable. Although the level of total cell phosphotyrosine is reduced, bone marrow-derived macrophages from *fgr-/-; hck-/-; lyn-/-* mice have no obvious defects in LPS-induced activation (Meng et al 1997).

Thy-1 In T cells, cross-linking of the GPI-linked protein Thy-1 also causes an increase in total cell phosphotyrosine, and at least two Src PTKs, Fyn and Lck, are implicated in this event (Stefanova et al 1991, Thomas & Samelson 1992). Fyn and Lck coprecipitate with Thy-1, and this interaction is dependent on the GPI anchor. In addition, thymocytes from *fyn-/-* mice are defective in Thy-1 signaling, indicating that Fyn plays a critical role in this GPI-linked receptor pathway (Lancki et al 1995).

F3 and F11/contactin Fyn has also been found to associate with F3 and contactin/F11, two other GPI-linked receptors in neurons (Olive et al 1995, Zisch et al 1995). F3 is a member of the immunoglobulin superfamily and is involved

in regulating neurite extension and repulsion. Fyn and the adhesion molecule L1 have been found to complex with F3 in neural tissues (Olive et al 1995). Because L1 is a transmembrane protein, it may be involved in linking Fyn to F3, but additional studies are necessary to address this possibility. Cross-linking of the GPI-linked cell adhesion molecule contactin/F11 also results in the coprecipitation of Fyn. In addition, there is an increase in total cell phosphotyrosine (Zisch et al 1995). Whether Fyn is involved in the biological events regulated by these GPI-linked receptors in vivo remains to be determined.

ASSOCIATION   Because Src PTKs are found on the inner face of the plasma membrane and on GPI-linked receptors on the outer leaflet, it is unclear how these molecules are linked to each other. One model proposes that a transmembrane coreceptor links the GPI and Src PTKs. A candidate bridging protein is an 85-kDa transmembrane protein found in Thy-1-Src PTK complexes (Stefanova & Horejsi 1991). A contactin-associated transmembrane protein, p190^{Caspr}, has recently been identified (Peles et al 1997). This protein contains a proline-rich sequence in its cytoplasmic domain that could mediate coupling with Fyn through the Fyn SH3 domain. In the case of the urokinase plasminogen activator receptor (uPA-R), two integrins, LFA-1 and CR3, have been shown to associate with this complex along with Src PTKs (Bohuslav et al 1995). Because Src PTKs have been linked to integrin signaling, it is possible that integrins could bridge GPI-linked receptors to Src family kinases.

A second model, based on studies on two GPI-linked proteins found in T cells, CD59 and CD55, suggests that Src PTKs colocalize with GPI-linked proteins. Colocalization is mediated by their N-terminal fatty acylation modifications (myristylation and palmitylation) (Shenoy Scaria et al 1993, Rodgers et al 1994). Mutations that prevent myristylation or palmitylation of Lck or Fyn abrogate their ability to interact with CD55 and CD59. In addition, Src, which is myristylated but not palmitylated, cannot interact with CD55 or CD59. Replacement of the first 10 amino acids of Src with the corresponding sequences in Fyn or Lck, or mutation of serine residue 3 or 6 in Src to Cys (residues that are palmitylated in Fyn), allows Src to couple with CD55 and CD59. These mutations allow palmitate addition to Src, which appears to be important for targeting Src PTKs to the same membrane subdomain where GPI-linked proteins are found. Thus rather than providing direct interaction, the lipid modification may colocalize Src PTKs and GPI-linked proteins in glycoprotein-rich membrane domains. In support of this hypothesis, Src PTKs and GPI-linked proteins can be dissociated by raising the temperature of the membranes from 4 to 37°C (Rodgers et al 1994). The change in temperature is sufficient to allow triton-solubilization of the membrane fraction containing GPI-linked proteins. This model does not completely rule out the possibility of a coreceptor. Thus

both interaction with a coreceptor(s) and localization of Src by lipid modifications may be important for mediating Src PTKs interactions with GPI-linked receptors. In addition, because cross-linking of GPI-linked receptors results in colocalization of numerous signaling molecules (e.g. G protein subunits and integrins), these molecules may also be important for connecting Src PTKs to GPI-linked receptors (Lisanti et al 1994, Bohuslav et al 1995, Solomon et al 1996).

ACTIVATION   As indicated above, cross-linking of GPI-linked proteins induces tyrosine phosphorylation of multiple cellular proteins and recruitment of Src PTKs; however, it is uncertain how these kinases are activated upon ligation of the GPI-linked receptors. It is possible that a tyrosine phosphatase may be brought to the GPI-linked receptor complex upon receptor ligation. Alternatively, recruitment of a coreceptor may provide a binding site for SH2 or SH3 domains, which would activate Src PTKs. Identifying potential coreceptors and the role of tyrosine phosphatases in GPI signaling may help to address these questions. It should be noted that in some T cells, glycolipid-enriched membrane (GEM) domains (which contain GPI-linked proteins) have been proposed to sequester Src PTKs from the tyrosine phosphatase CD45, keeping Src PTKs inactive (Rodgers & Rose 1996). Thus movement of SrcPTKs out of these domains could allow access to CD45 and result in their activation. Whether activation of Src PTKs by a GPI-linked receptor such as Thy-1 requires movement of these kinases out of GEM domains has not been determined.

Channels

Channels play important roles in regulating the influx and efflux of small molecules and ions that regulate cellular functions. Several voltage-gated and ligand-gated channels have been shown to couple with Src PTKs including K^+ channels, the IP_3 receptor and other Ca^{2+} channels, and glutamate, NMDA, and N-acetylcholine receptors (Zhao et al 1992, Rusanescu et al 1995, Swope et al 1995, Suzuki & Okumura Noji 1995, Calautti et al 1995, Holmes et al 1996, Jayaraman et al 1996, Yu et al 1997). The evidence that Src PTKs physically associate with some of these receptors and, under certain conditions, mediate tyrosine phosphorylation suggests that Src PTKs may regulate channel function. However, Src PTKs can also be activated by these channels, suggesting that Src PTKs or kinases participate in the regulation of cellular functions induced by these receptors. Thus Src kinases may be involved in both upstream and downstream regulation of channel activity.

Voltage-Gated Channels

Ca^{2+} CHANNELS   Regulation of intracellular and extracellular calcium levels is important for diverse biological responses including cell proliferation and differentiation. In PC12 cells, depolarization-induced neurite outgrowth requires

the function of a voltage-gated Ca^{2+} channel. Activation of this channel results in an increase in Src-specific activity, and expression of a dominant-interfering mutant of Src blocks depolarization-induced neurite outgrowth (Rusanescu et al 1995).

Primary keratinocytes exposed to Ca^{2+} undergo a differentiation program that includes growth arrest, expression of specific keratins, and formation of desmosomes (Hennings et al 1989, Hennings et al 1992, Dlugosz & Yuspa 1993, 1994). These events are associated with an elevation in the level of total cell phosphotyrosine-containing proteins including the v-Src substrate cortactin (Filvaroff et al 1990, 1994, Calautti et al 1995). In human keratinocytes, Ca^{2+} induces a rapid increase in Src-specific activity and association of Src with three cellular proteins (Zhao et al 1992). Interestingly, Yes-specific activity decreases upon Ca^{2+} treatment (Zhao et al 1993). In the mouse, Fyn has also been proposed to play a role in Ca^{2+}-mediated keratinocyte differentiation (Calautti et al 1995). Analysis of keratinocytes from *fyn*-deficient mice suggests that there is a defect in their differentiation response. These defects include a reduction in expression of specific differentiation markers (e.g. keratin 1 and filaggrin) and loss of cortactin tyrosine phosphorylation. In normal mouse keratinocytes, Ca^{2+}-induced activation of Fyn is observed, but the increase in Fyn-specific activity is only detected after 4 h. These results suggest that in the murine system, Fyn may not be functioning directly downstream of a Ca^{2+} channel. Instead, protein kinase C, which is regulated by intracellular Ca^{2+} concentrations, may be involved in Fyn activation. Treatment of keratinocytes with phorbol ester, an activator of PKC, results in a rapid (2 min) activation of Fyn-specific activity. The FAK-related PTK Pyk2 could also play a role in regulation of Fyn in mouse keratinocytes. Pyk2 is regulated by calcium and can associate with and activate Src PTKs (Lev et al 1995, Dikic et al 1996). Thus although Fyn does play a role in Ca^{2+}-induced differentiation of mouse keratinocytes, Fyn function/activation may not be directly coupled to a Ca^{2+} channel. Instead, downstream effectors like PKC or Pyk2 may be more directly involved in activation of Fyn.

It should be noted that in the keratinocyte system, Ca^{2+} may trigger some responses via effects on other cellular receptors. Ca^{2+}-induced tyrosine phosphorylation in keratinocytes has been linked to a channel; however, extracellular Ca^{2+} can also regulate cell adhesion molecules (Gumbiner 1993, Filvaroff et al 1994). For example, integrins and cadherins require Ca^{2+} for cell-cell and cell-matrix interactions. Because some adhesion molecules have been shown to activate Src PTKs, these receptors could also be involved in Src PTK activation in keratinocytes.

It is not known if, or, indeed, how these kinases are linked to Ca^{2+} channels and what the mechanism of activation might be. In human keratinocytes, where

rapid activation of Src is observed, the increase in kinase activity is accompanied by dephosphorylation of Src, suggesting that a tyrosine phosphatase is important for regulating Src function downstream of the Ca^{2+} channel (Zhao et al 1992). In mouse keratinocytes, Ca^{2+} induces dephosphorylation of the negative regulatory tyrosine of Fyn (Calautti et al 1995).

K^+ CHANNELS   Studies on voltage-dependent potassium channels suggest that Src PTKs can also regulate ion channel function. In T cells, tyrosine phosphorylation of the Kv1.3 K^+ channel correlates with an inhibition of channel function, and this phosphorylation is blocked in Jurkat cells, which lack Lck (Szabo et al 1996). Src can also associate with the human voltage-dependent potassium channel Kv1.5, in vivo and in vitro via the SH3 domain of Src and a proline-rich sequence in the receptor (Holmes et al 1996). In addition, co-expression of v-Src induces tyrosine phosphorylation of Kv1.5 and a decrease in channel activity. Whether such a modification occurs under normal physiological conditions has not been determined. It is also unclear whether tyrosine phosphorylation per se or binding of the Src SH3 domain with the Kv1.5 channel is responsible for suppression of channel function.

Ligand-Gated Channels

n-AChR   A ligand-gated channel, nicotinic acetyl choline receptor (n-AChR), can also interact with Src PTKs. In chromaffin cells, the n-AChR, which stimulates Ca^{2+} influx, is involved in release of catecholamines (Perlman & Chalfie 1977). Both serine/threonine and tyrosine kinases are involved in catecholamine secretion, and Fyn is activated following receptor stimulation (Ely et al 1994, Allen et al 1996). Because activation of Fyn is dependent upon Ca^{2+} influx, the Ca^{2+}-regulated kinase Pyk2 may play a role in the n-AChR-mediated Fyn activation (Lev et al 1995, Cox et al 1996).

Src PTKs also function in n-AChR-mediated membrane depolarization of neuromuscular junctions (Swope et al 1995). Membrane depolarization at neuromuscular junctions involves clustering of the n-AChR in response to neural proteoglycans such as agrin. Studies in the Torpedo electric organ have shown that the δ subunit of the AChR is tyrosine phosphorylated and associates with Fyn and a Fyn/Yes related kinase (Fyk) in response to agrin-induced clustering. This association is likely to be mediated by the SH2 domain because a Fyn or Fyk SH2 domain fusion binds the phosphorylated δ receptor subunit. In mammalian cells, the role of Src PTKs in n-AChR signaling is unclear. Tyrosine phosphorylation of the β and δ subunits has been observed; however, it is unclear if Src PTKs are associated with the receptor (Wallace et al 1991, Qu et al 1994). Studies in mice have implicated a third tyrosine kinase, MuSK, in tyrosine phosphorylation of the AChR (DeChiara et al 1996, Glass et al 1996).

MuSK is a RPTK that is complexed with an accessory protein found in myotubes. This complex can bind agrin, which results in the activation of MuSK and clustering of the AChR. Loss of MuSK or agrin prevents AChR clustering, which suggests that both proteins are required. In addition, MuSK activation correlates with phosphorylation of the β subunit of the AChR, and this phosphorylation is important for AChR aggregation. Because Src PTKs can couple to RPTKs, it is possible that Src PTKs may interact with MuSK and phosphorylate the AChR. *src-/-;fyn-/-* mice show no defects in agrin-induced clustering, suggesting that these two Src PTKs have either a redundant role or no role in ACHR clustering (S Burden, personal communication).

1,4,5 INOSITOL TRISPHOSPHATE (IP$_3$) RECEPTOR/Ca^{2+} CHANNEL   Another example of a channel that may couple to Src PTKs is found in T cells. Engagement of the TCR induces multiple cellular responses including increases in tyrosine phosphorylation and alterations in Ca^{2+} levels. Changes in Ca^{2+} concentration partly result from production of the second messenger IP$_3$. IP$_3$ binds to a receptor located on the endoplasmic reticulum (IP$_3$-R) (Harnick et al 1995). The IP$_3$-R is a ligand-gated calcium channel that, when activated, releases intracellular calcium. In T cells, stimulation of the TCR induces a physical association between the IP$_3$-R and Fyn and tyrosine phosphorylation of the receptor (Jayaraman et al 1996). Thymocytes from *fyn*-deficient mice have a reduction in IP$_3$-R tyrosine phosphorylation, which correlates with a defect in TCR-mediated Ca^{2+} release. Thus it is likely that Fyn may function in vivo to regulate this ligand-gated Ca^{2+} channel.

NMDA RECEPTOR   The NMDA receptor, a ligand-gated channel, plays an important role in neuroplasticity and synaptic transmission in the central nervous system (Hollmann & Heinemann 1994). Both serine/threonine and tyrosine kinases have been implicated in NMDA signaling pathways (Raymond et al 1993, Wang & Salter 1994, Wang et al 1996b). Two other Src family kinases, Src and Fyn, have been found to function downstream of the NMDA receptor (Grant et al 1992, Yu et al 1997). Src can coprecipitate with the NMDA receptor, and activation of Src PTKs by incubation with a high-affinity ligand for the SH2 domain results in an increase in channel activity (Yu et al 1997). This change in channel activity is dependent upon sequences in the unique domain of Src, and an antibody recognizing the unique domain of Src decreases NMDA channel gating. Thus Src interacts with the NMDA receptor or an associated protein through its unique domain, and this interaction is important for regulation of the receptor by Src. Since at least two of the receptor subunits are tyrosine phosphorylated, it is possible that the NMDA receptor may be a substrate of Src. Alternatively, a second Src PTK, Fyn might be involved in phosphorylating the receptor. Fyn has also been found to phosphorylate the NMDA receptor in

vitro and has been implicated in long-term potentiation (LTP), a process regulated by the NMDA receptor (Grant et al 1992, Grant & Silva 1994, Suzuki & Okumura Noji 1995). How Src PTKs are activated by the NMDA receptor is unclear, but these kinases appear to play a role in regulating this ligand-gated channel.

GAP JUNCTIONS   Gap junctions are specialized membrane structures that serve as intercellular channels to regulate cell:cell communication. Phosphorylation of the subunits of these junctions, referred to as connexins, regulates gap junctional communication (review, Lau et al 1996). Connexin43 is phosphorylated on tyrosine in v-src transformed cells, and this event correlates with a decrease in gap junction communication (Crow et al 1990, Filson et al 1990, Swenson et al 1990).

Although the role of c-Src in regulation of gap junctions by receptor pathways has not been determined, c-Src can phosphorylate connexin 43 in vitro on sites identical to those observed in vivo (Loo et al 1995). In addition, the cytoplasmic regions of connexin 43 contain proline-rich motifs and tyrosine phosphorylation sites that could provide docking sites for Src PTKs. Thus gap junctions may also be linked to Src PTKs. Although EGF and FGF treatment causes a reduction in junctional communication, connexin 43 is phosphorylated on serine, not tyrosine (Lau et al 1992, Doble et al 1996).

Although additional studies are needed to better define the link between Src PTKs and various channels, the above examples demonstrate that Src family kinases are likely to be involved in relaying signals to and from this class of receptors.

Stress Responses

Stress-inducing agents such as ultraviolet C (UVC) irradiation, heat, or hypoxia trigger signaling pathways that mediate either protection or killing of affected cells. The best-characterized cellular stress responses are those involved in the induction of changes in gene expression. For example, irradiation with short-wavelength ultraviolet light (UVC) induces activation of MAP kinases that activate pre-existing transcription factors (AP-1, TCF/elk-1, NF-κB), which mediate transcriptional activation of c-fos, c-jun, and other genes (Stein et al 1989, Rahmsdorf et al 1992, Devarey et al 1992, 1993, Radler-Pohl et al 1993). Ras and Raf are activated in this response and dominant-negative variants of these proteins block transcriptional activation (Devarey et al 1992, 1993, Radler-Pohl et al 1993). In addition, UVC treatment leads to induction of dimerization, tyrosine phosphorylation, and internalization of the EGF-R, and a truncated dominant-negative variant of the EGF-R blocks the UVC transcriptional response (Warmuth et al 1994, Miller et al 1994, Sachsenmaier et al 1994, Coffer et al 1995, Huang et al 1996).

c-Src has been implicated in the UVC transcriptional responses by the evidence that c-Src kinase activity is elevated after UVC treatment, and expression of kinase-inactive Src variants inhibits UVC-induced activation of Jun and NF-κB (Devarey et al 1992, 1993). Because Src phosphorylates the EGF receptor (Wasilenko et al 1991) and is associated with, and activated by, the EGF receptor (Oude Weernink et al 1994, Sato et al 1995a), it is possible that Src could act upstream or downstream of the EGF receptor in this response pathway. Likewise, Src could either directly mediate activation of Ras and Raf, or their activation could be dependent on EGF receptor phosphorylation.

EGF-R activation by UVC irradiation can be blocked by antioxidants, and the activation of EGF-R can be mimicked with H_2O_2, suggesting that reactive oxygen intermediates may be responsible for the activation of EGF-R following UVC irradiation (Huang et al 1996). Src family kinases have also been reported to be activated by H_2O_2 and other oxidants (Nakamura et al 1993, Hardwick & Sefton 1995, Gonzalez-Rubio et al 1996). For example, Lck is activated by H_2O_2 in T cells and fibroblasts. Lck activation is not associated with decreased phosphorylation on the C-terminal negative regulatory Tyr505; however, it is dependent on phosphorylation of the catalytic cleft activation loop tyrosine residue analogous to Tyr416 of Src (Hardwick & Sefton 1995). Hardwick & Sefton found that a kinase-inactive mutant variant of Lck is still phosphorylated following H_2O_2 treatment, and they proposed that another tyrosine kinase activated by H_2O_2 treatment may phosphorylate Lck on Tyr394 and activate its catalytic activity (Hardwick & Sefton 1995). Alternatively, H_2O_2 may inhibit a tyrosine phosphatase that constitutively dephosphorylates Y394. Src kinase activation by H_2O_2 treatment has not been detected in all cell types. Activation of Fyn, Lyn, and Lck was not detected in Ramos B cells exposed to H_2O_2 treatment under conditions where Syk was detectably activated (Schieven et al 1993). These results raise the possibility that oxygen intermediates activate Src kinases, which in turn phosphorylate the EGF receptor, leading to activation of Shc, Ras, and Raf. This pathway would mimic the proposed Src-mediated activation of EGF receptor by $G_{\beta\gamma}$-induced signaling pathways (Luttrell et al 1997).

Oxygen deprivation, or hypoxia, also induces changes in gene expression including an induction of the angiogenesis factor, vEGF, as well as endothelin, and PDGF B-chain (Kourembanas et al 1990, 1991). Treatment of cultured cells under hypoxic conditions causes an activation of Src and Fyn kinase activity, and kinase-inactive variants of Src block hypoxia-induced changes in vEGF mRNA accumulation (Mukhopadhyay et al 1995, Seko et al 1996a,b). These results suggest that Src may be involved in promoting angiogenesis in tumors by participating in the induction of vEGF; however, the mechanism for Src activation under these conditions has not been elucidated.

In PC12 cells, activation of stress pathways by sorbitol leads to activation of the FAK-related protein tyrosine kinase, Pyk2/RAFTK/CAKβ. Overexpression

of Pyk2 induces Jun-kinase (JNK) activation and a dominant-negative mutant of Pyk2 blocks stress-induced JNK activation (Tokiwa et al 1996). Because Src has been shown to couple with Pyk2 in other PC12 receptor pathways (Dikic et al 1996), it is possible that these two tyrosine kinases may cooperate in stress response pathways in certain cell types.

In summary, a variety of different stress conditions lead to activation of Src kinases. Changes in gene expression mediated by UVC treatment and hypoxia appear to be dependent on Src kinases; however, the precise mechanism that mediates Src activation is not understood, and the downstream effectors of Src activity involved in this response have not been definitively identified.

CELLULAR EVENTS REGULATED BY SRC KINASES

The preceding sections have discussed the activation of Src family kinases by diverse families of receptors that induce cellular responses that affect growth control, survival and differentiation, cytoskeletal arrangements, secretion, channel function and other biological activities. Many of the responses to a specific receptor overlap with those of unrelated receptors (e.g. induction of DNA synthesis), whereas other responses are relatively receptor-specific (e.g. neurite outgrowth). What receptor-mediated events are regulated by Src kinases, and do Src kinases mediate similar or distinct biological events when activated by different receptor classes? In this section, we address these questions by discussing potential effector functions of Src in these receptor pathways.

It is difficult to establish the precise functional roles of individual Src kinases in receptor pathways for many reasons: 1. Closely related kinases appear to play redundant roles in receptor pathways (e.g. the activation of Src, Fyn, and Yes by PDGF). 2. Expression of kinase-inactive, dominant-interfering variants of individual Src family members interferes with the function of multiple Src PTKs owing to the high degree of homology of the SH2 and SH3 domains of these kinases. 3. Src kinases can activate other families of protein tyrosine kinases (e.g. Syk/ZAP, Btk, RPTKs). Therefore, strategies to inhibit Src can block phosphorylation of substrates of downstream kinases. 4. It is difficult to directly correlate functions of mutant, constitutively activated kinases with those of their transiently activated cellular homologues. 5. The dependence of any one receptor on Src PTKs can vary in different cellular environments depending on whether redundant pathways can activate the same biological activity or on how strongly the receptor activates a Src PTK. 6. Small-molecule inhibitors that block the activity of specific PTKs have not been developed.

Src Kinase Substrates

An obvious approach to exploring cellular events regulated by Src PTKs is to identify substrates specifically phosphorylated by these enzymes following

Table 7   v-Src substrates phosphorylated after stimulation of cellular receptors

Substrates	Receptors
Cytoskeletal proteins	
Focal adhesion proteins	
FAK	Integrins, PDGF-R, insulin, NGF-R, EGF-R, $Fc\varepsilon RI$, bombesin-R, thrombin-R, bradykinin-R, endothelin-R, LPA-R, AngII-R, PAF-R, CCK-R, FMLP-R, gastrin-R
paxillin	PDGF-R, insulin-R, NGF-R, bombesin-R, PAF-R, ICAM, bradykinin-R, FMLP-R, gastrin-R, endothelin-R, LPA-R, AngII-R, CCK-R, TNFR-R, IL-3-R
p130^{cas}	Integrins, NGF-R, bradykinin-R, IL-8-R
talin	Thrombin-R, PDGF-R
tensin	Integrins
ezrin	Integrin, Met/HGF-R, CD4, CD3
Other actin cytoskeletal proteins	
cortactin, HS1	FGF-R, integrins, thrombin-R, calcium, TCR, ICAM
AFAP 110	
ezrin	Integrin, Met/HGF-R, CD4, TCR/CD3
Other structural proteins	
catenins (β, γ and p120)	CSF-1R, EGF-R, PDGF-R, Met/HGF-R
connexin 43	PDGF-R
caveolin	Insulin-R
calpactin	PDGF-R, EGF-R, insulin-R
tubulin	TCR, NFG-R
Enzymes	
Other protein tyrosine kinases	
Syk, /ZAP	IRRs, integrins, muscarinic-R, IL-2-R
Tec kinases	IRRs
FAK	See above
Protein serine/threonine kinase PKCδ	PDGF-R, EGFR, $Fc\varepsilon RI$, substance P-R
Enzymes involved in phospholipid metabolism	
PLC-γ,	NGF-R, PDGF-R, EGF-R
p85PI 3-K	Many RPTKs, thrombin-R, most IRRs, integrins, many class I cytokine receptors (not shown to be tyr phos in all these)
SHIP	FcγRI, BCR, m-CSF-R
smGTP-regulatory enzymes	
p190^{RhoGAP}	Many RPTKs, FcγRIIIA, BCR, TCR, receptors for bombesin, bradykinin, GM-CSF
p120^{rasGAP}	Many RPTKs, BCR, TCR

(continued)

Table 7   *(continued)*

Substrates	Receptors
Phosphatases	
SHP-1	Thrombin-R, NGF-R, insulin-R
SHP-2	PDGF-R, EGF-R, EPO-R, prolactin-R
PP2A	EGF-R
Adaptors	
Shc	Most RPTKs, many class I cytokine receptors, most IRRs, CD4, G_iPCRs
DOK (p62 GAP protein)	Many RPTKs, TCR, BCR, CD-2, high calcium
Cbl	TCR, BCR, EGF-R, FcRs
Receptors	
IRRs (ITAMs)	IRRs, CD2
Receptor PTKs	RPTKs, N-CAM, N-cadherin, G_iPCRs

receptor activation. The identification of candidate Src PTK substrates has been facilitated greatly by the analysis of constitutively activated variants of these kinases such as the v-Src transforming protein of RSV. Many candidate v-Src substrates have been identified by comparing the profiles of proteins phosphorylated on tyrosine in control and RSV-transformed cells. Table 7 shows some of the v-Src substrates that have been identified and a partial list of cellular receptors that stimulate tyrosine phosphorylation of these proteins. It is clear that many classes of receptors induce phosphorylation of proteins that are phosphorylated in v-Src-transformed cells, raising the possiblity that Src PTKs are involved in receptor-induced phosphorylation of these proteins. However, it is difficult to establish whether Src kinases are responsible for phosphorylation of these substrates following receptor activation (since multiple PTKs are activated by most receptor pathways) and to determine which cellular responses are regulated by each substrate phosphorylation event.

The properties of these substrates have been discussed extensively elsewhere (Kellie et al 1991, Schaller et al 1993, Brown & Cooper 1996). Since this review is focused on the biological events regulated by Src family kinases, we discuss these substrates only in the context of how they may be involved in specific receptor-induced events postulated to be regulated by Src kinases. Many of the substrates of Src PTKs are involved in the regulation of multiple biological activities. For example, PI 3-K has been implicated in the regulation of DNA synthesis, cell survival, differentiation, lamellipodia formation, chemotaxis and migration, adhesion, and neurite outgrowth (Vanhaesebroeck et al 1996). Likewise, phosphorylation of Shc and activation of the Ras-MAP kinase (MAPK) pathway are involved in induction of DNA synthesis, migration, oocyte maturation, mitosis, and neurite outgrowth. Thus a discussion of Src PTK effectors

involved in biological activities regulated by these kinases is complicated by the pleiotropic activities of Src, as well as many of its substrates.

It is noteworthy that recent studies of the function of Src in integrin signaling and in osteoclasts strongly suggest that the noncatalytic domains of Src also have important effector functions (Kaplan et al 1995, Schlaepfer et al 1997; P Schwartzberg, L Xing, B Boyce & HE Varmus, unpublished results). These studies indicate that the SH2- and SH3-binding domains of Src kinases (and possibly the unique domain) may serve adaptor-type functions independent of catalytic activity.

Adhesion and Spreading

The role of Src in adhesion mediated by integrin receptors for the extracellular matrix protein fibronectin has been examined in *src-/-* fibroblasts. These fibroblasts display a reduced rate of adhesion and spreading on a fibronectin matrix, suggesting that Src is required for optimal adhesion efficiency but is not essential for these events (Kaplan et al 1995). *src-/-* fibroblasts could not be distinguished from Src-expressing fibroblasts on a collagen matrix, indicating that Src may function specifically in fibronectin receptor adhesive responses.

Overexpression of Csk has also been used as a strategy to examine the role of Src in cell adhesion. Overexpression of Csk in HeLa cells causes a conversion of these cells to a spherical, loosely adhered morphology and changes the morphology of $\alpha_V\beta_5$ integrin structures on the ventral cell surface. This activity was dependent on the catalytic activity of Csk. Because Csk overexpression can inactivate c-Src by maintaining a high stoichiometry of Y527 phosphorylation, these data provide additional supportive evidence for a role of Src in cell adhesion and spreading. The role of Src in regulating cell adhesion is not known. However, Src catalytic activity does not appear to be critical for this event because the defect in cell adhesion in *src-/-* fibroblasts can be rescued with Src mutants lacking the catalytic domain (Kaplan et al 1995). Rescue requires an intact SH2 and SH3 domain. These results suggest that Src may serve as an adaptor protein to localize specific proteins to adhesive structures (possibly focal adhesions) involved in cell adhesion and spreading.

Focal Adhesion Formation/Disassembly

Focal adhesions and related structures are induced following engagement of many integrins. These structures are specialized membrane-attachment plaques where integrins couple the extracellular matrix with bundled actin cable filaments and complex assemblies of other cytoskeletal proteins (Jockusch et al 1995, Brown & Cooper 1996, Burridge & Chrzanowska-Wodnicka 1996). Focal adhesions are important for cell adhesion, morphology, and cell migration. Fyn

and Yes are also expressed in fibroblasts and may serve redundant functions. The evidence that many v-Src substrates, including paxillin, p130^{cas}, talin, vinculin, tensin, FAK, and β1 integrin subunit are associated with focal adhesion sites and phosphorylated on tyrosine suggests that Src family kinases may be involved in focal adhesion dynamics (Bockholt & Burridge 1993, Jockusch et al 1995, Schwartz et al 1995, Clark & Brugge 1995, Petch et al 1995, Bhattacharya et al 1995, Brown & Cooper 1996, Burridge & Chrzanowska-Wodnicka 1996).

Tyrosine phosphorylation appears to be involved in the formation of focal adhesions because PTK inhibitors block this event; however, since multiple tyrosine kinases are associated with focal adhesions (Src, Fyn, FAK, Abl), it is not known which kinases are critically involved (Burridge et al 1992, Romer et al 1992, 1994, Seckl & Rozengurt 1993, Ridley & Hall 1994, Chrzanowska-Wodnicka & Burridge 1994, Lewis et al 1996). It is likely that the major role for tyrosine phosphorylation in focal adhesion formation is the induction of protein interactions through creation of SH2-binding sites. However, tyrosine phosphorylation may also regulate the activity of enzymes involved in focal contact assemblies.

The role of Src PTKs in focal adhesion assembly/disassembly is not understood. Src-/- fibroblasts are able to assemble focal adhesions, indicating that Src does not serve a unique function in focal adhesion formation. Fibronectin-induced tyrosine phosphorylation of p130^{cas} is defective in fibroblasts from Src-deficient mouse embryos (Bockholt & Burridge 1995, Hamasaki et al 1996, Vuori et al 1996, Schlaepfer et al 1997). In contrast, cells deficient in FAK, Fyn, or Abl are not defective in phosphorylation of p130^{cas} (Bockholt & Burridge 1995, Hamasaki et al 1996). Because p130^{cas} does not localize to focal adhesions in Src-/- fibroblasts and because kinase-dead Src variants can rescue p130^{cas} localization to focal adhesions and tyrosine phosphorylation, it appears that Src is involved in the recruitment of p130^{cas} to focal adhesions, rather than being directly responsible for its phosphorylation (Schlaepfer et al 1997; K Vuori, unpublished results).

As described above, Src and Fyn bind to FAK and may participate in FAK-mediated signaling events by phosphorylating FAK (thus creating sites for recruitment of proteins to FAK complexes) and, possibly, by regulating FAK kinase activity (Schaller et al 1994, Cobb et al 1994, Xing et al 1994, Eide et al 1995, Calalb et al 1995, 1996, Schlaepfer & Hunter 1996). The role of FAK in focal adhesion assembly/disassembly is not definitively established; however, several lines of evidence indicate that FAK plays an important role in cell migration. It has been postulated that the defect in cell migration in FAK-/- fibroblasts is the result of a defect in disassembly of focal adhesions.

Structures similar to focal adhesions are assembled by integrins in other cell types. Src, Yes, talin, vinculin, spectrin, and other focal adhesion proteins

are associated with large, detergent-insoluble complexes involved in $\alpha_{IIb}\beta_3$-mediated platelet aggregation (Horvath et al 1991, Fox et al 1993). Src-deficient platelets do not show any detectable defects in platelet aggregation; however other Src related kinases may compensate for Src since Yes, Fyn, and Lyn are also recruited to integrin complexes in aggregated platelets (Fox et al 1993).

Lamellipodia

Sheet-like protrusions of actin filaments assembled in a meshwork are referred to as lamellipodia or membrane ruffles. v-Src induces a rapid induction of membrane ruffling within minutes after it is activated [using conditional mutants of v-Src (Boschek et al 1981)]. The substrates involved in these microfilament reorganizations are not known; however, candidate effector proteins include PI 3-K, ezrin, vinculin, cortactin, p190 Rho^{GAP}, and AFAP, which are phosphorylated on tyrosine following stimulation by many classes of receptors (Table 7). The involvement of c-Src or other family members in receptor-induced lamellipodia or other changes in cortical actin-associated structures is not well established. However, Src kinases and several substrates have been implicated in these events by the evidence described below.

PI 3-K regulates the small GTP-binding protein Rac, a potent inducer of membane ruffling (Kotani et al 1994, Wennstrom et al 1994, Nishiyama et al 1994, Ridley et al 1995). Membrane ruffling induced by insulin, IGF-1, scatter factor/HGF, and PDGF is blocked by inhibitors of PI 3-K. Because activated variants of Src activate PI 3-K activity, it is possible that Src-mediated activation of PI 3-K is involved in membrane ruffling induced by certain classes of cellular receptors.

p190^{RhoGAP} is phosphorylated in v-Src-transformed cells and growth factor-stimulated cells (Ellis et al 1990, Bouton et al 1991, Settleman et al 1992). This protein associates with p120^{RasGAP} through p120^{RasGAP} SH2 domains and displays Rho and Rac GTPase-activating activity (McGlade et al 1993, Ridley et al 1993, Chang et al 1995). Its potential involvement in Src-mediated actin rearrangements was highlighted by the evidence that actin stress fiber dissolution and EGF-induced condensation of p190 and p120^{RasGAP} into cytoplasmic, arc-like structures (which delimit regions of greatest stress fiber dissolution) was inhibited in cells overexpressing a kinase-inactive form of Src (Chang et al 1995). These effects correlated with decreased phosphorylation of p190^{RhoGAP}. Conversely, overexpression of kinase-active Src increased the rate of appearance and number of cells exhibiting EGF-induced arcs as well as tyrosine phosphorylation of p190^{RhoGAP} (Chang et al 1995). These results suggest the possibility that p190 phosphorylation by Src following EGF receptor activation activates Rho GAP activity of p190, which leads to the early dissolution of actin filaments.

Cortactin is a 80–85 kDa F-actin cross-linking protein enriched in cortical structures such as ruffles and lamellipodia (Wu & Parsons 1993, Huang et al 1997). It is phosphorylated in v-Src-transformed cells, as well as in cells treated with various growth factors, thrombin, or with integrin or ICAM ligands (Table 7) (Maa et al 1992, S Wong et al 1992, Fox et al 1993, Wu & Parsons 1993, Durieu-Trautmann et al 1994, Zhan et al 1994, Vuori & Ruoslahti 1995, Ozawa et al 1995, Bhattacharya et al 1995, Calautti et al 1995). Cortactin redistributes with actin into podosomes after phosphorylation in Src-transformed cells; however, the function of cortactin and the effects of tyrosine phosphorylation are not understood (Wu & Parsons 1993, Okamura & Resh 1995). In vitro phosphorylation of cortactin by c-Src causes a dramatic inhibition of its F-actin cross-linking activity and moderate inhibition of F-actin binding (Huang et al 1997). Studies of cells from csk-/- mice suggest that cortactin may be a preferred substrate of Src (relative to Fyn) and that there is a correlation between stress fiber dissolution and cortactin hyperphosphorylation (Thomas et al 1995). In csk-/- or csk-/-; fyn-/- fibroblasts, cortactin is hyperphosphorylated and localized to podosomes and actin stress fibers are lost. However, cortactin is not hyperphosphorylated in csk-/-; src-/- fibroblasts, its localization resembles wild-type fibroblasts, and stress fibers are partially intact.

FGF receptor-induced tyrosine phosphorylation of cortactin takes place 4–8 h after treatment (at the mid-late G1 phase of the cell cycle). This event is coincident with Src activation, and Src coprecipitates with cortactin, suggesting that Src may be responsible for FGF-induced cortactin phosphorylation (Zhan et al 1993, 1994).

Recent studies of the pathogenic bacteria Shigella flexneri suggest an involvement of Src and cortactin in actin-cytoskeletal rearrangements that are involved in bacterial invasion (Dehio et al 1995). Invasion of cells by S. flexneri occurs via bacterium-directed phagocytosis, which involves actin polymerization at the bacterial entry site. Src is recruited to the entry structure and to the periphery of the phagosome. Bacterial invasion is associated with increased levels of cortactin phosphorylation, which is enhanced in cells overexpressing c-Src. Cortactin is highly enriched in the entry structures and in the periphery of the phagosome.

Ezrin is a widespread protein localized to an actin-containing membrane skeleton. It is a member of the ERM (ezrin/radixin/moesin) family of proteins, which are capable of mediating membrane-cytoskeletal interactions through their amino-terminal talin/band 4.1 domain and their C-terminal actin-binding domain (Tsukita et al 1997, Gould et al 1989, Turunen 1989, Algrain et al 1993). The actin-binding site of ezrin is masked in the intact protein and is believed to be uncovered by conformational changes that relieve intramolecular interactions between the N- and C-terminal halves of ezrin. v-Src phosphorylates ezrin on

two tryrosine residues located in each of the domains of ezrin (Krieg & Hunter 1992). It is possible that tyrosine phosphorylation may affect the intramolecular interactions of ezrin or interactions of ezrin with actin or the plasma membrane. Overexpression of the C-terminal portion of ezrin leads to the formation of lamellipodia (Martin et al 1995), and ezrin antisense oligonucleotides decrease filopodia and lamellipodia formation (Takeuchi 1994).

Ezrin localizes to membrane ruffles induced by EGF (Bretscher 1989). Ezrin is phosphorylated on tyrosine after engagement of scatter factor (Met) receptor PTK, EGF (Bretscher 1989), and cross-linking of either CD4 or CD3 (Bretscher 1989, Thuillier et al 1994, Jiang et al 1995). Although Src family kinases are activated by all these receptors, the involvment of Src family kinases in ezrin phosphorylation was investigated in only one study. Ezrin phosphorylation induced by cross-linking of CD4 was blocked by mutations in CD4 that prevent interaction with Lck (Thuillier et al 1994).

Taken together, these studies provide substantial support for the possibility that Src is involved in receptor-mediated phosphorylation of cytoskeletal proteins that affect cortical actin cytoskeletal rearrangements; however, the precise effects of phosphorylation on these proteins and their role in cytoskeletal rearrangements remain to be determined.

Migration

Cell migration is induced following treatment with a variety of agonists that mediate movement of cells in tissue development, wound healing, lymphocyte homing, tumor metastasis, and other cellular functions. Many signaling molecules can influence cell migration through effects on cell adhesion (affecting either affinity/avidity for adhesive ligands and/or cytoplasmic stabilization of adhesion), cytoskeletal rearrangements involved in dissociation and spreading, or expression of adhesion receptors or signaling molecules. Preliminary findings in several experimental systems suggest that Src may be involved in cell migration. v-Src has been shown to induce migration of PC12 pheochromocytoma cells in combination with PMA (similar to the way NGF and PMA induce migration) (Altun-Gultekin & Wagner 1996).

Fibroblasts from src-/- mice display a significantly slower rate of locomotion than wild-type fibroblasts, and this defect is rescued by expression of a kinase-active, but not inactive c-Src variant (Hall et al 1996). In addition, expression of a dominant-negative Src inhibits locomotion that is mediated by the RHAMM-receptor for hyaluronan, and c-Src associates with RHAMM (Hall et al 1996).

c-Src is also implicated in migration induced by EGF in a rat carcinoma cell line. c-Src is activated when NBT-II rat carcinoma cells are treated with EGF when subconfluent (Rodier et al 1995). Under these conditions, the cells undergo

epithelial-to-mesenchyme transition, dissociate from cell clusters, and become motile. Cell dissociation and scattering induced by EGF is inhibited following microinjection of kinase-inactive Src, and overexpression of c-Src causes a subpopulation of cells to undergo spontaneous cell dissociation and to display increased sensitivity to EGF. c-Src has also been implicated in endothelial cell migration induced by inhibition of angiotensin II and by the associated stimulation of u-PAR activity (Bell et al 1992). Lastly, migration of T cells induced by lymphocyte chemoattractant factor (LCF) requires CD4 and Lck (Ryan et al 1995). It is of interest that the catalytic activity of Lck is not required for this response. Therefore, CD4-Lck is required for LCF-induced motility; however, this response is independent of the enzymatic activity of Lck. The events downstream of CD4-Lck coupling have not been identified. Src could be acting at many differents steps in the pathways leading to cell migration, possibly through effects on integrin-mediated adhesion (potentially involving FAK), rearrangements of cortical actin-cytoskeleton involved in cell spreading, or changes in gene expression that affect expression of integrins, other adhesive receptors, or downstream signaling molecules.

MAPK has recently been shown to be essential for integrin-induced cell migration. Because Src has been implicated in integrin-induced MAPK activation, it is possible that the role of Src PTKs in cell migration is partially mediated by activation of MAPK (Schlaepfer et al 1997, Klemke et al 1997). In addition, FAK has also been shown to be involved in integrin-mediated cell migration. Overexpression of FAK stimulates cell migration, and this process is defective in cells lacking FAK or expressing FAK-dominant inhibitory variants (Wilson et al 1995, Ilic et al 1995, 1996, Cary et al 1996, Gilmore & Romer 1996). FAK mutants lacking the Src-binding site Y397 do not enhance cell migration when overexpressed in fibroblasts, suggesting that Src, Fyn, or another protein that binds to this phosphorylation site (e.g. PI 3-K) are required for FAK's role in cell migration (Cary et al 1996, Chen et al 1996). Lastly phosphorylation of PI 3-K by Src could also be involved in Src-mediated migration because PI 3-K appears to be involved in regulating cell migration (Bornfeldt et al 1995, Yokote et al 1996).

Cell Cycle Progression

INDUCTION OF DNA SYNTHESIS   The ability of constitutively activated forms of Src family members to induce DNA synthesis and cell proliferation implies that activation of the wild-type kinases can stimulate pathways leading to cell proliferation. However, constitutive activation of Src kinases by mutations does not necesssarily mimic activation mediated by receptor-stimulated pathways where these kinases are generally activated only transiently. Therefore, one cannot conclude that activation of Src kinases by cellular receptors is sufficient

to stimulate cell proliferation. More likely, Src kinases contribute to a whole program of events that can lead to stimulation of DNA synthesis and cell cycle progression under natural, receptor-driven activation.

Src family kinases appear to be required for induction of DNA synthesis by several growth factor receptor protein tyrosine kinases. Microinjection of DNA encoding a kinase-inactive form of Src or Fyn (Twamley-Stein et al 1993) or a neutralizing antibody that recognizes a conserved sequence in the C-proximal tail of Src, Fyn, and Yes inhibits induction of DNA synthesis by EGF, PDGF, and CSF-1. In addition, expression of a kinase-inactive or kinase domain-deleted Src mutant in src-/- fibroblasts blocks PDGF induction of DNA synthesis (Broome & Hunter 1996). The SH2 domain of Src is required for association with the PDGF receptor; however, the SH3 domain appears to play an important role in PDGF- and EGF-induced DNA synthesis because mutant forms of Src carrying mutations in the SH3 domain dominantly interfere with the induction of DNA synthesis by these growth factors (Broome & Hunter 1996, Erpel et al 1996).

Recent evidence suggests that the induction of *myc* transcription is the PDGF-β pathway dependent on Src/Fyn/Yes (Barone & Courtneidge 1995). PDGF induction of *myc* is blocked by Src-inhibitory antibodies, and the antibody-induced block in DNA synthesis can be rescued with exogenous expression of *myc*. While Src is activated by bombesin treatment of Swiss 3T3 cells (Rodriguez-Fernandez & Rozengurt 1996), Src does not appear to be a critical player in bombesin-induced DNA synthesis because the Src-neutralizing antibodies were ineffective in blocking this event.

Src appears to be specifically involved in *myc* activation in PDGFβ receptor pathways; however, other receptors that activate Src may utilize this kinase for activation of distinct cellular pathways known to be involved in the induction of DNA synthesis. For example, v-Src activates MAP kinase and PI 3-K, signaling molecules known to participate in receptor-induced cell proliferation. The evidence implicating Src kinases in the phosphorylation of a few representative signaling molecules that are involved in receptor-induced DNA synthesis is discussed below.

Shc   Tyrosine phosphorylation of Shc stimulates the Ras-MAP kinase pathway, which is critically involved in proliferation induced by v-Src and many proliferative receptors. Shc phosphorylation is detected following engagement of growth factor receptors, immune response receptors, GPCRs, cytokines, integrins, and some stress pathways. Microinjection of anti-Shc antibody inhibited DNA synthesis induced by insulin, IGF-1, and EGF, but not by serum (Sasaoka et al 1994). The induction of Shc phosphorylation by stress inducers and GPCRs may be mediated by RPTKs since these kinases are activated during

these responses, and dominant-negative, kinase-inactive EGFR can block activation of the MAP kinase pathway by GPCR agonists (Miller et al 1994, Coffer et al 1995, Daub et al 1996). Are Src family kinases involved in Shc phosphorylation by any of these receptors? Because Shc can directly interact with most receptor tyrosine kinases, it is unlikely that Src is involved in phosphorylation of Shc and activation of the Ras-MAP kinase pathway by these receptors. However, Src may be involved directly or indirectly in Shc phosphorylation by the other receptors. Src family kinases coprecipitate with Shc after stimulation of certain receptor pathways (Ptasznik et al 1995, Luttrell et al 1996), suggesting that Src kinases may directly phosphorylate Shc. Integrin-mediated Shc phosphorylation in certain cell types was recently shown to be independent of the α or β receptor cytoplasmic tails, and dependent on an interaction with another transmembrane protein, possibly caveolin (Wary et al 1996). Caveolin has been shown to interact with Src, thus this kinase or related PTKs could be involved in integrin-mediated Shc phosphorylation (Li et al 1996b). As described above, activation of Shc and EGFR tyrosine phosphorylation through $G_{\beta\gamma}$ subunits in COS7 cells is dependent upon the c-Src protein tyrosine kinase, and $G_{\beta\gamma}$ expression leads to the formation of Shc-Src and Shc-EGFR complexes (Luttrell et al 1996, 1997). These results suggest that $G_{\beta\gamma}$ activation of Src PTKs is essential for Shc phosphorylation, most likely through recruitment of Shc to the EGFR via interaction of the Shc SH2 or PTB domain with Src-dependent phosphorylation sites on the EGFR.

p85:p110 PI 3-K   This PI 3-K isozyme is a heterodimeric enzyme consisting of a p85 regulatory subunit and a p110 catalytic subunit, which phosphorylates the D3 position of phosphatidylinositol (Carpenter & Cantley 1996). It is activated by v-Src, RPTKs, class I cytokines, G protein–coupled receptors, and other receptors. Activation of this isoform is mediated through interaction with the SH2 and SH3 domains of the p85 regulatory subunit, as well as through Ras interactions with p110. Recently, the oncogene of an avian retrovirus has been shown to encode a PI 3-K catalytic domain, indicating that under certain conditions, this enzyme is sufficient for constitutive stimulation of DNA synthesis (Chang et al 1997).

The role of PI 3-K in the induction of DNA synthesis varies in different cell types and for different receptors. For example, p85:p110 PI 3-K has been shown to be required for PDGF, but not CSF-1 (which does not induce D3 polyphosphatidylinositol accumulation), induced DNA synthesis (Roche et al 1995a). As with Shc, most RPTKs contain binding sites for p85 and do not appear to be dependent on Src for activation of this lipid kinase. However, the Src kinases activated by immune response receptors can be coprecipitated with p85, suggesting that Src may contribute to p85 phosphorylation (Augustine et al

1991, Yamanashi et al 1992, Pleiman et al 1993). GPCR activation of the MAPK pathway is inhibited by the PI 3-K inhibitor wortmannin, at a step upstream of tyrosine phosphorylation of Shc, indicating that PI 3-K (possibly a different isoform, γPI 3-K) is required for activation of the PTK that phosphorylates Shc (Hawes et al 1996). Because Shc phosphorylation by $G_{\beta\gamma}$ requires Src (Luttrell et al 1996), PI 3-K may be linked with the Src pathway in this system.

Growth factor receptors   As described above, v-Src has been shown to phosphorylate several growth factor receptors (Wasilenko et al 1991, Peterson et al 1994, Stover et al 1995). These studies raise the possibility that c-Src is involved in RPTK activation by other receptor pathways. For example, thrombin-induced IGF-1R phosphorylation, ET-1-, LPA-, and thrombin-induced EGFR phosphorylation, and angiotensin II-induced PDGF-R phosphorylation could all involve c-Src. This possibility is supported by the evidence that c-Src is activated by all these agonists and that c-Src is required for $G_{\beta\gamma}$-induced activation of EGFR phosphorylation (Rao et al 1995, Linseman et al 1995, Daub et al 1996, van Biesen et al 1996, Luttrell et al 1997). Thus c-Src-mediated RPTK activation may be involved in the mitogenicity of some agonists that do not directly interact with receptor PTKs.

All these studies indicate that Src may participate in receptor pathways leading to the stimulation of DNA synthesis through a variety of mechanisms including, but not limited to, phosphorylation of Shc, leading to activation of MAP kinase pathway; activation of PI 3-K; and activation and/or phosphorylation of RPTKs.

G2/M TRANSITION   Src is also involved in regulating a second stage of the cell cycle involving the G2/M phase transitions. The first hint that Src regulates events taking place during the G2/M phase of the cell cycle came from studies in *Xenopus* showing that v-Src accelerates meiotic maturation. However, it was not established whether this effect resulted from activation of the Ras/MAP kinase pathway or from other effector pathways operative during meiosis (Spivack et al 1984, Spivack & Maller 1985). More definitive evidence for a role for c-Src in somatic cell mitosis has been obtained from studies of Src in cells arrested during mitosis using the metaphase inhibitor, nocodozole, or in cells microinjected with Src inhibitory antibodies (Shalloway & Shenoy 1991, Fumagalli et al 1994, Taylor & Shalloway 1996). Src activity is elevated several-fold in metaphase-arrested cells, and this increase in activity correlates with Src phosphorylation by Cdc2 kinase at several serine and threonine residues in the N-terminal unique region. These and other studies suggest that phosphorylation by Cdc2 initiates a process leading to Src activation during mitosis. Roche and coworkers have further shown that Src inhibitory antibodies microinjected into G2 phase mouse fibroblasts blocked subsequent cell division prior to nuclear

envelope breakdown (Roche et al 1995a). Src activity was required throughout G1 phase for induction of DNA synthesis. These results suggest that Src is involved in a function critical for progression from G2 to cell division. This function appears to be redundant with those of Fyn and Yes, which are also activated during mitosis, because an antibody that recognizes Src alone did not prevent cell division—only the cross-inhibitory anti-COOH peptide antibodies were effective.

One mitosis-specific v-Src substrate that could mediate Src effects during mitosis is p68^{sam} (G Wong et al 1992, Taylor & Shalloway 1994, Courtneidge & Fumagalli 1994). This protein binds to Src via SH3 and SH2 domain interactions and shares significant homology with gld-1, Grp33 and shaking (Weng et al 1994, Taylor & Shalloway 1994, Jones & Schedl 1995, Ebersole et al 1996). All four proteins contain a KH RNA binding motif, which is located in the middle of the 170 amino acid homology region (referred to as GSG domain). HnRNP K, which contains three KH domains, also binds to the Src SH3 domain through a proline-rich motif similar to those in p68^{sam}. The gld-1 protein from *Caenorhabditis elegans* plays a negative regulatory role in mitotic activity during pachytene of oocyte meiotic prophase (Francis et al 1995a) and a non-essential role in negatively regulating proliferation of premeiotic germ cells (Francis et al 1995b). Loss of gld-1 function results in exit from pachytene, return to the mitotic cycle, and the development of tumors. Mutations in the KH domain show loss of function phenotypes, suggesting that this domain is required for tumor suppressor activity. It is tempting to speculate that p68^{sam}, like gld-1, negatively regulates mitotic progression. Tyrosine phosphorylation of p68^{sam} may inhibit its function and relieve this negative control. However, there are notable differences between p68^{sam} and gld-1 that raise doubts about whether these proteins share identical functional activities (no SH3 binding motifs in gld-1, no homology outside the GSG domain, nuclear localization of gld-1 not detected). Because the KH domain is involved in RNA and single-stranded DNA binding, the function of these KH motif proteins may be involved in RNA- or DNA-dependent binding events. p68^{sam} has been shown to bind to RNA, and this binding is lost following tyrosine phosphorylation by Src (G Wong et al 1992, Taylor & Shalloway 1994, Wang et al 1995). Recently, HnRNP K was shown to have strand-specific single-stranded DNA binding activity and to act as a transcriptional activator, raising the possibility that other KH proteins also regulate transcription (Wang et al 1995, Michelotti et al 1995, 1996, Tomonaga & Levens 1996, Lee et al 1996).

It is possible that Src may regulate G2/M effectors other than Sam68, thus affecting alterations in the cytoskeleton or adhesion that take place during mitosis. Alternatively, Src kinases may be involved in activation of the Ras-MAP kinase pathway during mitosis. Raf has been shown to be activated during

mitosis (Laird et al 1995, Pathan et al 1996). In T cells, Raf binds to a mitosis-specific form of Lck that migrates with a retarded electrophoretic mobility. Mitotic activation of Raf was not observed in the Lck-deficient JCaM1.6 cell line, suggesting that Lck is involved in this cell-cycle specific activation of Raf (Pathan et al 1996). The Mos-MAPK pathway has been implicated in various aspects of mitotic and meiotic activities in *Xenopus* and mouse oocytes, at least partially related to spindle formation and polar body degradation (Minshull et al 1994, Gotoh & Nishida 1995, Choi et al 1996).

Apoptosis

The viability of vertebrate cells depends on signals transduced by survival factors that suppress apoptosis. Growth factors and cytokines, as well as extra-cellular matrix and cell-cell interactions, provide these signals that determine the fate of a cell to either survive (and possibly proliferate) or to undergo apoptosis. Signals transduced through a single receptor, e.g. the TCR or sIgM, can promote either apoptosis or cell proliferation depending on the way in which the cell is programmed to respond to receptor-proximal activation events.

Expression of v-Src has been shown to rescue several cell types from apoptotic death induced by either removal of cytokines, irradiation, chemotherapeutic drugs, or disruption of cellular interactions with extracellular matrix proteins (anoikis) (Anderson et al 1990, McCubrey et al 1993, Frisch & Francis 1994, Canman et al 1995, Basu & Cline 1995). These results suggest that constitutively activated Src can mimic the effects of cytokine receptors, integrins, and other receptor pathways that protect cells from certain programs of apoptosis. Studies in hematopoietic cells have provided evidence that the cellular homologues of Src kinases are involved in either protection from apoptosis or the induction of apoptosis.

TCR induction of cell death through the Fas pathway appears to be mediated through transcriptional induction of Fas ligand. Lck is required for induction of Fas ligand expression; however, the apoptotic pathway activated following Fas ligand binding to its receptor is independent of Lck (based on evidence that Lck-deficient T cells undergo Fas-induced cell death with efficacy similar to that of Lck-positive T cells) (Schraven & Peter 1995, Oyaizu et al 1995, Latinis & Koretzky 1996, Gonzalez-Garcia et al 1997). However, *fyn*-deficient mice are less sensitive to killing by anti-Fas antibody and Fas-ligand cytotoxic T cells, which suggests that Fyn may play an important role in Fas signal transduction (Atkinson et al 1996). sIgM-induced apoptosis is inhibited by anti-sense Blk in CH31 B-lymphoma cells (Yao & Scott 1993). Lyn and Fgr appear to prevent apoptosis during retinoic acid-induced granulocytic differentiation of HL-60 cells (i.e. *lyn* or *fgr* anti-sense oligodeoxynucleotides undergo premature apoptosis) (Katagiri et al 1996). In contrast, Lyn anti-sense oligonucleotides

reverse the cell survival advantage provided to neutrophils by GM-CSF (Wei et al 1996). In addition, cross-linking of CD4 or stimulation by HIV-interaction with CD4 can lead to apoptosis (Corbeil et al 1996). This response requires the CD4 Cys-X-X-Cys, which is involved in Lck interaction. Introduction of Lck into Lck-deficient CD4+ T cells greatly increased HIV-induced apoptosis and syncytium formation. The apoptotic response did not require the kinase activity of Lck, suggesting that Lck may function as an adaptor in coupling with apoptotic signals.

These studies in hematopoietic cells suggest that Src kinases may play a role in regulating apoptosis; however, the precise mechanisms whereby Src exerts an effect in these cells remain to be established. The role of Src kinases in some of these systems could be indirect, involving coordination of initial events that trigger receptor-proximal signaling events (e.g. Lck phosphoryation of ITAMs in the TCR to initiate T cell responses). Alternatively, Src kinases could affect apoptotic responses through stimulation of pathways leading to MAP kinases or PI 3-K activation. Current studies indicate that the fate of a cell to either survive (and possibly proliferate) or to undergo apoptosis is dependent on the strength and balance of signals leading to activation of the various MAP kinase pathways (Erks, JNK/Sap-1s/p38Sap2s) as well PI 3-K (Yao & Cooper 1995, Xia et al 1995, Cuvillier et al 1996, Kauffmann-zeh et al 1996, Verheij et al 1996, Gardner & Johnson 1996, Johnson et al 1996, Park et al 1996a, Frisch et al 1996, Graves et al 1996, Minshall et al 1996, Wilson et al 1996, Kulik et al 1997). Thus to the extent to which Src kinases can regulate the activation of Erks and PI 3-K, these PTKs may participate in cellular pathways that regulate cell survival. Further studies are required to define the precise steps leading to or preventing apoptosis that are controlled by these kinases.

Differentiation

Expression of v-Src in immature cells can induce dramatic changes in the differentiation program of the cells. In most cell types, v-Src expression blocks cell differentiation. For example, infection of avian myoblasts, retinoblasts, or chondroblasts with RSV maintains these cells in a proliferative state and blocks differentiation into myotubes, neuroretinal cells, epidermal cells, or chondrocytes, respectively (Muto et al 1977, Yoshimura et al 1981, Crisanti-Combes et al 1982, Alema & Tato 1987). In contrast, introduction of v-Src into PC12 cells or immature sympathetic neurons induces neurite outgrowth and terminal differentiation into neuron-like cells (Alema et al 1985, Haltmeier & Rohrer 1990, Hecker et al 1991). As discussed previously, these dramatic alterations in cell differentiation induced by v-Src do not imply that cellular Src mediates these effects when activated under natural conditions. Although Src kinases may be involved in some of these responses under natural conditions, it is likely that

v-Src, as an unregulated, promiscuous PTK, mimics the cellular activities of other protein tyrosine kinases through phosphorylation of common substrates, and that the nature of the cellular responses to v-Src is dependent on how the cell is programmed to respond to the signals activated by high level, sustained phosphorlyation of these substrates.

A comparison of the biochemical responses of v-Src and NGF in PC12 cells indicated that many of the changes induced by v-Src mimic those of NGF, including phosphorylation of cellular proteins, induction of gene transcription, and priming to NGF (Rausch et al 1989, Thomas et al 1991). Together, these results suggest that v-Src's ability to function as an inducing agent is a consequence of its ability to mimic critical aspects of the NGF differentiation program. However, c-Src would appear to be a critical component of NGF's actions because microinjection of anti-Src monoclonal antibodies blocks NGF-induced neurite outgrowth (Kremer et al 1991). Thus as in the example of proliferative responses to PDGF, CSF-1, and EGF receptors, c-Src appears to contribute to the cellular responses mediated by these RPTKs, but the activation of Src by these receptors would not appear to be sufficient for induction of either cell proliferation or differentiation.

The ability of v-Src to block differentiation of immature cells raises the question of whether the block in differentiation is indirectly a consequence of the stimulation of cell proliferation or whether it can alter transcription of differentiation-specific genes independently of cell proliferation. In myoblasts, this issue has been addressed by activating a temperature-sensitive mutant of v-Src following myotube differentiation. Expression of v-Src led to inhibition of transcription of many muscle-specific genes in the differentiated myotubes in the absence of an induction of cell proliferation, which suggests that Src is able to inhibit the myogenic transcriptional program via a more direct mechanism (Schneider & Olson 1988, Falcone et al 1991). v-Src also induces transcription of many differentiation-specific genes in fibroblasts and other cell types, e.g. αD globin and keratin 18 (Pankov et al 1994, Itoh-Lindstrom & Leffak 1994). These results indicate that activated variants of Src can feed into transcriptional control pathways that regulate cell differentiation, thus raising the possibility that cellular homologues of these genes can participate in the pathways as well.

The role of Src kinases in differentiation of hematopoietic cells has been examined very thoroughly through studies of mice lacking single kinases or combinations of different kinases. For an extensive description of these studies and a discussion of the IRR and MHC receptors, see Lowell & Soriano (1996).

Gene Transcription

Many of the biological activities discussed in this section involve changes in gene transcription. Studies of v-Src in fibroblasts have defined many cellular

genes whose transcription is positively or negatively regulated by v-Src expression (Bedard et al 1989, Jahner & Hunter 1991, Qureshi et al 1991, Qureshi et al 1992, Frankfort & Gelman 1995, Scholz et al 1996). v-Src transcriptional activation involves multiple transcriptional control elements, including NF-κB, ATF/CRE, AP-1, CArG, and SIE sites (Dehbi et al 1992, Eicher et al 1994, Xie et al 1994, Simonson et al 1996, Cao et al 1996). The best example of a receptor-mediated transcriptional event regulated by c-Src would be the control of *myc* transcription by the PDGFβ receptors (Barone & Courtneidge 1995). Inhibition of c-Src with an inhibitory antibody blocked activation of *myc* transcription. Endothelin-induced *fos* induction also appears to be regulated by Src because expression of a kinase-inactive Src mutant blocks the endothelin induction of this gene (Simonson et al 1996).

IN VIVO BIOLOGICAL ACTIVITIES OF SRC KINASES

It is clear from the discussion above that Src PTKs couple with a plethora of receptors, engage unique and common sets of targets, and participate in the regulation of diverse biological responses. Most of the studies discussed involved analysis of immortalized cell lines or isolated primary cells. Examining the functions of these kinases in whole organisms is important for understanding how these molecular and cellular activities relate to both normal and disease-associated physiological events that involve Src PTKs.

Understanding the normal physiological role of Src PTKs has been aided by the advent of gene targeting and embryonic stem cell technology in the mouse. In addition, studies in *Drosophila* have aided in understanding Src PTK functions in vivo. Targeted disruptions of all known mammalian Src PTK genes have been generated in mice. The phenotypes of these mutant mice range from no overt defects to very distinct abnormalities in specific cell types, tissues, or physiological responses. For a detailed summary of these phenotypes, see reviews by Lowell & Soriano 1996 and Brown & Cooper 1996. The section below focuses on how the phenotypic effects of *src, fyn,* and *lck* disruption in mice relate to the roles of these kinases in the receptor pathways covered in this review.

Effects of Src Disruption in Mice

As indicated above, disruption of Src function in the mouse results in osteopetrosis, a bone remodeling defect (Soriano et al 1991). A more severe form of osteopetrosis is seen in *hck/src* double disruption mutants (Lowell et al 1996b). While the osteopetrotic phenotype of *src-/-* mice was an unexpected finding (given the predicted role of Src in cell proliferation and the absence of evidence suggesting a role for Src in bone physiology), subsequent studies revealed that

Src is expressed at very high levels in osteoclasts, cells involved in bone remodeling (Horne et al 1992). Hck is also present at high levels in osteoclasts (Lowell et al 1996b). Bone remodeling involves a dynamic balance between bone resorption and bone deposition. Osteopetrosis results when this balance is perturbed either through decreased resorption or excess deposition. In src-deficient mice, osteopetrosis is the result of a defect in bone resorption mediated by osteoclasts. Osteoclasts are present in the src-deficient mice (in fact, in excess); however, these cells display severe defects in bone resorption in vivo and in vitro (Boyce et al 1992, Lowe et al 1993). Bone resorption by osteoclasts involves the formation of a ruffled border, which secretes proteases, hydrogen ions, and other bone hydrolyzing agents at the site of osteoclast adhesion to bone. Formation of the ruffled border is defective in src-/- osteoclasts (Boyce et al 1992).

The molecular basis for this phenotype is not understood; however, the osteoclast resorption defect could involve alterations in at least two different receptor pathways. Attachment of the osteoclast to the bone is thought to be mediated by an interaction between the ECM molecule, osteopontin (OPN) and its receptor, the $\alpha_V\beta_3$ integrin. Src has been found to co-immunoprecipitate with the $\alpha_V\beta_3$ integrin and is also activated after OPN treatment (Rolnick et al 1992, Hruska et al 1995, Chellaiah et al 1996). Thus loss of Src could affect OPN or other matrix signaling responses necessary for the final maturation of osteoclasts following adhesion to bone. Interestingly, analysis of OPN expression in osteoclasts from Src-deficient mice shows a decrease in the level of OPN protein (Chackalaparampil et al 1996). Thus Src function may affect both ligand production and signaling by this adhesion receptor.

Alternatively, RPTK pathways may be impaired by loss of Src. Osteoclasts express several RPTKs, including the CSF-1 receptor and two HGF family members (Grano et al 1996, Kurihara et al 1996). Loss of the CSF-1 gene in mice causes osteopetrosis. However, this phenotype is the result of a defect in osteoclast differentiation, rather than osteoclast function (Yoshida et al 1990). Because Src PTKs can associate with the CSF-1 receptor and are activated after CSF-1 treatment (Courtneidge et al 1993), it is possible that Src is required for specific functions of the CSF-1 receptor that are necessary for resorption activity in mature osteoclasts but are dispensable during osteoclast maturation. CSF-1 treatment of osteoclasts induces cell spreading, an induction of total cell phosphotyrosine, and activation of Src (Insogna et al 1997). Src appears to be important for CSF-1 signaling in osteoclasts because src-/- osteoclasts do not spread in response to CSF-1. Thus defects in CSF-1 signaling could contribute to the osteoclast defect in src-/- mice.

A second family of RPTKs, the HGF/SF receptor family, may also play a role in osteoclast function. Osteoclasts express at least two HGF receptor

family members, STK and Met (Grano et al 1996, Kurihara et al 1996). STK is a receptor for macrophage stimulating protein (MSP). MSP treatment of osteoclast-like cells (derived by culturing of murine bone marrow in the presence of 1,25-dihydroxyvitamin D3 and interleukin-3) results in formation of a ruffled border and redistribution of Src to these borders. This correlates with an increase in bone resorption (Kurihara et al 1996). The ligand (HGF) for the closely related family member Met is secreted by osteoclasts. HGF treatment of osteoclasts increases Src kinase activity and alters osteoclast cell shape (Grano et al 1996). Thus defects in these RPTK pathways owing to the loss of Src could play a role in the osteopetrotic phenotype of src-/- mice. Analysis of whether loss of Src affects ligand secretion and/or signaling by these receptors should lead to insight into the molecular and cellular basis for the defects observed in Src-deficient mice.

Osteoclasts from src-/- mice display decreased levels of total cell phosphotyrosine-containing proteins relative the src+/+ osteoclasts (P Schwartzberg, HE Varmus, unpublished results). Because several downstream targets of Src such as FAK, tensin, cortactin, and PI 3-K are likely involved in osteoclast function, decreased tyrosine phosphorylation of some of these proteins could be involved in the osteoclast defect (Berry et al 1994, Hiura et al 1995, Hruska et al 1995) . Cbl tyrosine phosphorylation is impaired in src-/- osteoclasts and in wild-type osteoclasts treated with src anti-sense oligonucleotides (Tanaka et al 1996). Although tyrosine phosphorylation of other proteins has not been carefully examined in src-/- osteoclasts, cortactin, tensin, and FAK are found in the ruffled borders of osteoclasts, lacking src-/- osteoclasts (Berry et al 1994, Hiura et al 1995). In addition, PI 3-K is associated with the $\alpha_V\beta_3$ integrin and there is an increase in D3 phosphatidylinositols in OPN-treated osteoclasts (Hruska et al 1995).

Although some of these proteins may be direct substrates of Src, transgenic studies suggest that Src has a kinase-independent function in osteoclasts. Expression of wild-type, autophosphorylation-deficient, or a kinase-inactive transgene in src-/- mice can rescue the osteopetrotic defect (P Schwartzberg, L Xing, B Boyce & HE Varmus, unpublished results). Analysis of tyrosine phosphorylation indicates that the expression of kinase-inactive Src restores tyrosine phosphorylation, although there may be some differences in the overall profile of proteins phosphorylated. These results suggest that direct phosphorylation by Src of a subset of proteins is unlikely to be required for osteoclast function. Instead, Src may be important for regulating the localization of certain proteins or stabilizing signaling complexes that are necessary for formation of the ruffled border and bone resorption. Consistent with this hypothesis, Cbl, a scaffolding protein that binds to multiple signaling proteins, is not localized properly in Src-deficient osteoclasts (Tanaka et al 1996). In addition, in avian

osteoclasts, Src appears to associate with microtubules, and this association is not dependent upon tyrosine phosphorylation (Abu-Amer et al 1997). Because the microtubule network is important for transport of proteins to the ruffled border, it is possible that loss of Src could affect microtubule-based protein trafficking in osteoclasts.

Whether impairment in integrin, RPTK, or other receptor pathways is responsible for the osteopetrotic phenotype in Src-deficient mice is unclear. However, these studies, together with previous work on Src PTKs, provide some testable hypotheses for understanding the molecular basis for Src function in vivo.

Effects of Fyn and Lck Disruption on T Cell Development in Mice

The role of Fyn and Lck in T cell development and activation has been examined through analysis of mice that either overexpress mutant or wild-type forms of these kinases or are deficient in expression of these proteins due to disruption of the endogenous genes. Twentyfold overexpression of FynT in mice leads to enhanced TCR responses in thymocytes (Cooke et al 1991). In contrast, overexpression of a kinase-inactive form of FynT significantly inhibited TCR-mediated activation in otherwise normal thymocytes. Thymocytes from mice lacking either FynT or FynT plus the ubiquitously expressed form of Fyn (FynB) show significant reductions in calcium and proliferative responses to stimulation through the TCR. However, peripheral T cells re-acquired significant signaling responses ($\approx$50% of the wild-type response), and splenic T cells displayed only a partial or undetectable response to alloantigen (Appleby et al 1992, Stein et al 1992). These results suggest that the functions of Fyn in TCR stimulation are redundant in peripheral T cells, but to a much less significant extent in thymocytes.

Although disruption of Fyn does not affect maturation of thymocytes, Lck disruption results in a significant reduction in the number of CD4+/CD8+ thymocytes, thus Lck may play a critical role in early thymocyte maturation (Molina et al 1992, Lewin et al 1993, Penninger et al 1993). However, small populations of CD4+/CD8+ thymocytes can develop into mature $\alpha\beta$ T cells that are partially responsive to TCR-mediated stimulation (Molina et al 1992, Penninger et al 1993, Kawai et al 1995). Peripheral T cells from mice lacking Lck show severe defects in TCR signaling by antigen, however the same cells could be stimulated by anti-CD3, suggesting that Fyn is able to compensate for the absence of Lck when a large population of TCR/CD3 is engaged under receptor cross-linking conditions. The functional redundancy of Lck and Fyn in T cell signaling and maturation was further supported by the demonstration that disruption of both the Fyn and Lck genes completely arrests $\alpha\beta$ T cell development at the CD4-/CD8-stage (van Oers et al 1996b, Groves et al 1996). In addition, expression of an activated FynT transgene can restore production

of mature CD4+/CD8+ double-positive thymocytes and $\alpha\beta$ cells and improve representation of CD4+ and CD8+ single-positive cells in Lck-deficient mice (Groves et al 1996). These results suggest that Lck is the primary Src-related kinase involved in thymocyte maturation but that Fyn can partially compensate for Lck, albeit much less effectively. This conclusion is supported by the evidence that the constitutive ζ phosphorylation found in fresh thymocytes is severely reduced in thymocytes from Lck-/- mice (van Oers et al 1996a).

Is the prominent role of Lck in early T cell development a consequence of a unique function of this kinase or of its specific expression in these cells? Although differences in the levels of expression could contribute to the different dependencies on Fyn and Lck, several lines of evidence suggest that the functional activities of these kinases are distinct (Olszowy et al 1995). As discussed above, kinase-inactive Lck disrupts thymocyte development, whereas kinase-inactive Fyn does not, and expression of active Lck causes thymic tumors, whereas Fyn only induces hyperstimulatable T cell responses (Anderson & Perlmutter 1995). Fyn can be coprecipitated or cocapped with the TCR, whereas Lck is not detectable under similar conditions (Samelson et al 1990, 1992, Gassman et al 1992), and Lck couples with CD4 and CD8 whereas Fyn does not. In addition, differential roles of FynT and FynB were inferred from the ability of activated variants of FynT, but not FynB, to cause an increase in TCR-stimulated IL-2 production (despite similar hyper-responsiveness as measured by induction of tyrosine phosphorylation) (Davidson et al 1992). FynT was also shown to be more efficient in promoting antigen receptor-triggered calcium fluxes, and calcium ionophores partially rescue FynB's inability to enhance antigen-mediated lymphokine secretion (Davidson et al 1994). The distinct catalytic domain sequences of FynT, not the distinct SH2 sequences, are responsible for the improved ability to augment antigen responsiveness and calcium mobilization. Thus the unique structural features of FynT found within the N-proximal region of the cataltyic domain are responsible for the specific effects of this Fyn isoform in T cell signaling. It will be of interest to determine whether this difference affects substrate specificity of Fyn.

Effects of Fyn Disruption on Neural Functions

Loss of Fyn in mice also causes defects in neural functions, although the molecular basis for the neurological impairments is less well understood. Loss of Fyn has been associated with abnormalities in the hippocampus, defects in long-term potentiation (LTP), reduced myelination, increased fear response, and decreases in the rate of amygdala kindling (Grant et al 1992, Umemori et al 1994, Cain et al 1995). Although little is known about the molecular basis for amygdala kindling, some molecular events involved in LTP and myelination have been identified.

HIPPOCAMPUS/LTP   Disruption of the *fyn* gene results in abnormalities in hippocampal histomorphology manifested by an increase in granular cells in the dentate gyrus and pyramidal cells in the CA3 region (Grant et al 1992). Whether loss of Fyn increases the rate of cell proliferation, alters cell fate decisions, or inhibits apoptosis is unclear. Since Fyn has been linked to apoptosis in T-cells (see IRR section), it is possible that decreased apoptosis may contribute to the hippocampal defects (Atkinson et al 1996). In order to understand how loss of Fyn affects hippocampal development it is critical to determine what factors are important for generation and maintenance of these cell populations.

The abnormalities in the hippocampal architecture could contribute to the defects in LTP, which are also observed in *fyn-/-* mice. The hippocampus is important for memory and learning, and these processes have been linked to LTP. The impaired LTP in *fyn-/-* mice has been detected in the synapses formed by the CA3 pyramidal cells and the CA1 neurons of the hippocampus. Thus it is possible that the increase in pyramidal cell number is responsible for this defect as well (Grant et al 1992). However, the hippocampal architectural defects can be distinguished from the LTP defect because expression of a *fyn* transgene in the forebrain can rescue the LTP defect but does not correct the altered hippocampal morphology (Lowell & Soriano 1996). Therefore, a second potential explanation for the LTP phenotype observed in *fyn-/-* mice is a defect in the neurotransmitter signaling pathway activated at the synapse. The major neurotransmitter found at the CA3/CA1 synapse is L-glutamate, which binds to NMDA and non-NMDA receptors on the post-synaptic CA1 neurons (Collingridge et al 1983). Activation of the NMDA receptors initiates a cascade of signaling events, including changes in intracellular calcium and activation of serine/threonine kinases and tyrosine kinases (Grant & Silva 1994). Recently, Src was shown to interact directly with the NMDA receptor via its unique domain, and antibodies to Src are able to decrease channel gating (Yu et al 1997). Both Src and Fyn phosphorylate the NMDA receptor and this phosphorylation is likely important for receptor function (Suzuki & Okumura Noji 1995, Yu et al 1997). Because there are no obvious defects in LTP in neurons of Src-deficient mice, these results suggest that Src phosphorylation and association with the receptor is not important for LTP (Grant et al 1992). It is possible that events downstream of the NMDA receptor are more severely affected in Fyn-deficient mice. A decrease in FAK tyrosine phosphorylation has been observed in the hippocampus of Fyn-deficient mice, but whether FAK plays any role in NMDA signaling is uncertain (Grant et al 1995). Defects in LTP have also been observed when NCAM function or Ca^{2+}/calmodulin-dependent kinase II (CamKII) function is blocked (Grant & Silva 1994, Mayford et al 1995, Bach et al 1995, Ronn et al 1995, Glazewski et al 1996). Both molecules have been proposed to function downstream of the NMDA receptor. Given that neurons

from Fyn-deficient mice are impaired in their ability to extend processes on NCAM-expressing fibroblasts, it is possible that analogous defects in the hippocampus may contribute to LTP impairment (Beggs et al 1994). Similarly, although there is no direct link between CamKII and Fyn, it is possible that loss of Fyn could be affecting CamKII signaling pathways. CamKII is activated by intracellular Ca^{2+} (Hanson & Schulman 1992). In endothelial cells, activation of CamKII-dependent signaling pathways precedes thrombin-induced tyrosine phosphorylation (Marsen et al 1995). As mentioned above, Ca^{2+}-induced differentiation of mouse keratinocytes results in Fyn activation (Calautti et al 1995). Thus loss of Fyn could affect CamKII signaling, which could contribute to the LTP defects. Determining how loss of Src and Fyn affects NMDA receptor tyrosine phosphorylation and NMDA signaling pathways should elucidate whether defects in this receptor signaling pathway are linked to the impairment of LTP in Fyn-deficient mice.

FEAR RESPONSE   An increased fear response has also been observed in Fyn-deficient mice (Miyakawa et al 1996). This defect has been observed in mice carrying an in-frame insertion of a β-galactosidase (lacZ) gene downstream of sequences in Fyn encoding the SH3 domain. This results in the production of a protein composed of the Fyn unique and SH3 domains fused to lacZ. The fear response has not been measured in *fyn* null mice, so this defect could be specific to this partial mutation. In any case, these results suggest that there may be multiple behavioral abnormalities due to loss of Fyn.

MYELINATION DEFECTS   In addition to the architectural abnormalities in the hippocampus and the impaired LTP, some Fyn-mutant mice also show reduced myelination (Umemori et al 1994). The defect was originally observed in the in-frame lacZ mice described above. Analysis of myelination has not been done in *fyn* null mice; thus it is difficult to predict whether this defect results from the loss of Fyn. It should be noted, however, that Fyn can associate with myelin-associated glycoprotein (MAG) (Jaramillo et al 1994). MAG is an adhesion molecule implicated in myelination. Cross-linking of MAG induces a rapid increase in total cell phosphotyrosine and also activates Fyn. Thus defects in myelination observed in these Fyn mutants may be explained by disruption of MAG signaling.

Effects of Disruption of Both Src and Fyn in Mice

Given the large number of receptors to which Src PTKs couple, it is somewhat surprising that mutations in Src PTKs are tolerated during embryogenesis; however, it is also clear that multiple Src PTKs are involved in each receptor pathway. Thus the lack of a phenotype in mice containing a single Src gene disruption likely is due to the functional redundancy in tissues where these

kinases are highly expressed and where multiple Src PTKs are activated by the same receptors. Consistent with this hypothesis, more severe defects are observed when mutations are made in multiple Src PTKs (Lowell & Soriano 1996). Mutations in more than one Src PTK may be necessary in order to reveal a requirement for these kinases in particular systems. The more severe T cell defect in *fyn-/-;lck-/-* mice and the osteoclast defect in *hck-/-;src-/-* mice are consistent with this hypothesis (van Oers et al 1996b, Lowell et al 1996b). *fyn-/-;src-/-* mice die perinatally (Stein et al 1994). Although the basis for the lethality is unclear, detailed analysis of these mice may reveal requirements for Src PTKs in additional receptor pathways in vivo and provide insight into the molecular basis for the phenotype.

Drosophila Src Kinases

One of the surprising findings from studies of *src-/-* mice was the absence of any neuronal phenotype (Lowell & Soriano 1996). As mentioned in the introduction, Src is expressed at high levels in brain, and there are two alternatively spliced, neuron-specific forms of Src, referred to as Src(+). Closely related alternatively spliced mRNAs of Src have also been identified in *Xenopus laevis*. There are two Src-related genes in *X. laevis*, and proteins encoded by alternatively spliced Src mRNAs have a 5-amino acid insert. In addition, expression of these isoforms is observed after neural induction but before differentiation (Collett & Steele 1993). Although the evolutionary conservation of the alternatively spliced form of Src suggests that it is important, no definitive role for Src(+) has been found. Mammalian cell culture studies, however, suggest that c-Src is required for neurite outgrowth in response to various differentiation agents (Kremer et al 1991). Although it is possible that Src or Src(+) are not important for neural function in vivo, it is likely that the role of this kinase is masked by the presence of at least three other Src PTKs. Consistent with this idea, studies in *Drosophila* suggest that Src may be important in the nervous system in this organism. Three Src-related genes have been identified in *Drosophila* (Simon et al 1985, Gregory et al 1987, Takahashi et al 1996). Dsrc29 is more closely related to the Tec family kinases (Gregory et al 1987). The remaining two Src-related molecules, Dsrc64 and Dsrc41, share homology with Src throughout their sequences (Simon et al 1985, Takahashi et al 1996). Overexpression of these Src homologues in *Drosophila* embryos results in distinct consequences (Kussick et al 1993, Takahashi et al 1996). Dsrc41 overexpression has no effect on *Drosophila* development, whereas overexpression of wild-type Dsrc64 in *Drosophila* embryos is lethal, and expression in specific neuronal precursor cells of the eye disrupts eye development (Kussick et al 1993, Takahashi et al 1996). Biochemical analysis of Dsrc64 reveals that it is phosphorylated on the negative regulatory tyrosine as well as on the

positive regulatory autophosphorylation site, suggesting that this kinase may be activated during embryonic development, either through a receptor pathway or through incomplete phosphorylation by the *Drosophila* Csk homologue (Kussick et al 1993, Takahashi et al 1996). Thus similar to higher eukaryotic Src family kinases, these kinases may have slight differences in their regulatory mechanisms.

Expression of kinase-inactive Dsrc64 or Dsrc41 in certain neuronal precursors of the *Drosophila* eye disturbs normal eye development, which suggests that Src may play a role in neural development (Kussick et al 1993, Takahashi et al 1996). Although a similar phenotype has not been observed in flies that are null for Dsrc64 (see below), the presence of at least one other Src-related gene, Dsrc41, could account for the difference in phenotype.

Studies of flies expressing kinase-defective and -activated Dsrc41 in the neuronal precursor cells of the eye have also provided some clues to downstream pathways regulated by *Drosophila* Src (Takahashi et al 1996). Expression of an activated form of Dsrc41 (Dsrc41YF) results in an increase in the number of R7 photoreceptor cells. This phenotype is dependent on two receptor tyrosine kinases, Sevenless and the *Drosophila* EGF-R, which suggests that Dsrc41 interacts with RPTK pathways (Cagan 1993, Takahashi et al 1996). Defects in the formation of adherens junctions and organization of the actin cytoskeleton in preclusters are observed when the kinase-inactive mutant is expressed in precursors of R3 and R4. Specifically, there is a loss of actin fibers and cadherin localization to the cell-cell junctions between the R3 and R4 precursors. This phenotype is enhanced when there is a single dose reduction of the endogenous Dsrc41. These results suggest that Dsrc41 may regulate the cytoskeleton and cell-cell interactions.

Further support for a role for Src PTKs in cytoskeletal organization comes from analysis of *src64* null flies (M Simon, personal communication). Generation of a mature oocyte involves coordinated changes in the cytoskeleton. Female flies homozygous for a null mutation in Dsrc64 have a partial infertility defect that may be linked to oocyte development. *Drosophila* ovaries are composed of egg chambers; within each chamber are 15 nurse cells and a single oocyte. The cytoplasm of these cells is connected by structures called ring canals. These structures allow transport of nutrients from the nurse cells to the oocyte, and in one of the latter stages of development of the oocyte, the transfer of the cytoplasm of the 15 nurse cells into the single oocyte. This transfer results in expansion of the oocyte and shrinking of the nurse cells (Cooley & Theurkauf 1994). Dsrc64 null females have smaller eggs, and the eggs that do not produce larvae are unfertilized. The decreased egg size likely results from incomplete transfer of the nurse cell cytoplasms to the oocyte. The defect in transfer could be due to alterations in the ring canal. Ring canals of Dsrc64 null

females are smaller and occasionally become detached. In addition, whereas the ring canals normally stain with phosphotyrosine antibodies, ring canals from Dsrc64 null females are devoid of phosphotyrosine epitopes (M Simon, personal communication).

The lack of fertilization may also be linked to the ring canals/nurse cells. The micropyle structure is important for sperm entry. This structure is derived from border cells that migrate from the anterior pole of the egg chamber between the nurse cells to the nurse cell-oocyte border (Lehman 1995). The presence of aberrant nurse cells could perturb border cell migration, thereby affecting formation of the micropyle structure. Using sensitized genetic screens, dominant mutations have been identified that enhance the phenotype of a Dsrc64 weak allele. Interestingly, one of these mutations occurs in the Dsrc29 gene, which is most closely related to the tec family kinase BTK (M Simon, personal communication).

Thus studies on the *Drosophila* Src PTKS suggest that, similar to their mammalian counterparts, *Drosophila* Src-related kinases can interact with other PTK families and regulate similar activities (adhesion/cytoskeleton).

SUMMARY

The experimental studies discussed above clearly indicate that Src kinases are versatile enzymes that play key roles in regulating many biological activities induced by numerous cellular receptors. A central question raised by the promiscuity of these kinases is how signals that activate Src kinases can mediate distinct biological events following activation by different receptors. The same question could be raised for many different receptor-activated signaling enzymes, e.g. Ras, PI 3-K, protein kinase A and PKC. Like Src kinases, these enzymes are activated by a broad spectrum of receptors and have been implicated in regulation of many biological events. Thus the question can be more broadly stated: How does each receptor elicit distinct cellular responses utilizing a similar set of signaling molecules? Current evidence indicates that there are several factors that affect the biological consequences of activation of a specific signaling protein.

1. Qualitative and quantitative aspects of protein expression: Each cell expresses a unique set of specific isoforms of each class of protein and different levels of expression of each protein. Further specificity can be derived from alternative splicing of transcripts to alter the activity or coupling of signaling proteins.

2. Cellular localization: Each receptor has evolved unique strategies to recruit specific sets of proteins to signaling complexes. Thus, any one particular
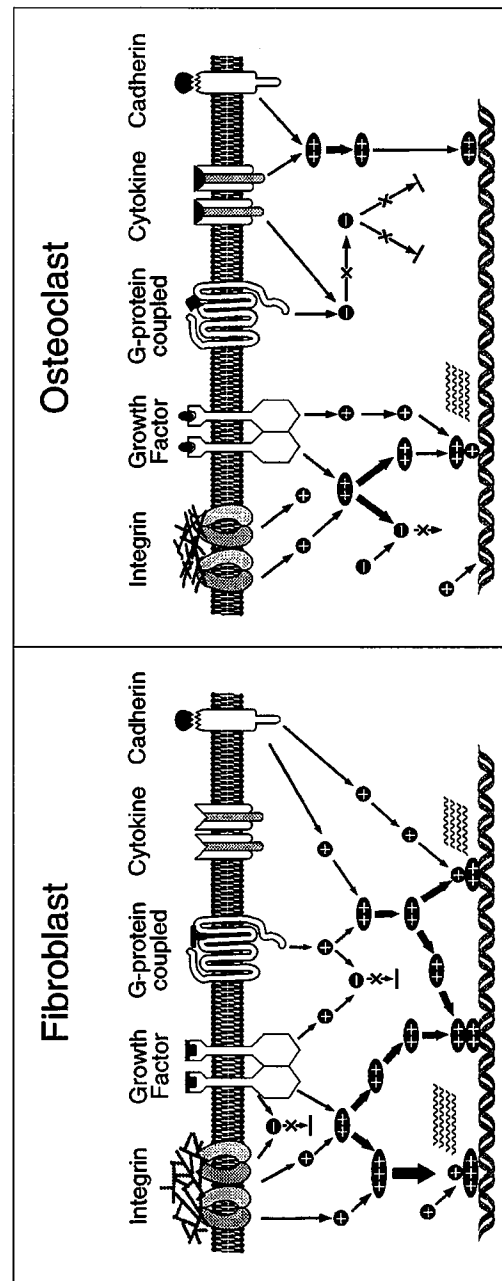
Figure 7 Combinatorial signal transduction.

enyzme may be in contact with, or proximal to, distinct effector molecules following activation by different receptors. Src PTKs and other signaling enzymes contain multiple binding domains allowing these proteins to couple with many other proteins that determine their subcellular compartmentalization.

3. Combinatorial effects: Because any one cell expresses multiple receptors that are in an activated or basal state under different conditions and because each of these receptors can cross-talk with other receptor signaling pathways through interactions with common downstream effectors, the strength of any one signal and its duration of action can be strongly influenced by synergistic or antagonistic influences from other receptor pathways.

Receptor signal transduction pathways are often compared to electrical circuitry networks, where the eventual outcome is dependent not only on the set of individual switches incorporated into the circuit board, but also on the combination of signals that are on or off at any one time. As illustrated in Figure 7, the factors described above all contribute to the combinatorial effects that allow Src to control bone resorption in an osteoclast and DNA synthesis in a fibroblast. The ultimate goal of research in this area is to be able to draw a complex circuitry scheme that explains how signals are processed in each cell. While reaching this goal may be far off, current research elucidating how proteins couple in distinct receptor pathways is laying the framework for future studies of how signals emanating from many different receptor pathways integrate with each other to control cell behavior.

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